From the Institute of Environmental Medicine
Karolinska Institutet, Stockholm, Sweden

# CHARACTERIZATION OF DIOXIN-INDUCED BONE TISSUE MODULATIONS: INVESTIGATING THE ROLE OF AHR AND THE RETINOID SYSTEM

Maria Herlin


Karolinska Institutet

Stockholm 2013

Published by Karolinska Institutet. Printed by Universitetsservice US-AB.


ISBN 978-91-7549-203-2

# ABSTRACT

All individuals are exposed to a large number of chemicals from multiple sources, and concern is growing that many everyday chemicals, alone or in combination, contribute significantly to observed increases in public health diseases. Bone tissue has been identified as a target for effects of environmental chemicals, however, possible consequences for human and wildlife health as well as the underlying mechanisms behind these effects are not yet well known.

In the present thesis, bone tissue modulations following exposure to dioxins were characterized in experimental models, and the role of a functional aryl hydrocarbon receptor (AhR) for the observed effects, as well as for a normal bone phenotype, was investigated. The results show that exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affects bone tissue in terms of altered geometrical, micro-structural, material and macro-mechanical properties. Osteoblast differentiation processes are affected by TCDD-exposure *in vitro*, which probably reflects one important cause for the disturbances of bone mineralization observed following *in vivo* exposure. Altered geometrical as well as densitometrical and micro-structural bone properties were associated with changes in circulating retinoid levels, which may reflect part of the observed bone modulations. Furthermore, altered expression of retinoid-related genes, as seen in osteoblastic cells following TCDD-exposure *in vitro,* might be a contributing mode-of-action underlying the disturbed osteogenesis process following dioxin exposure. A functional AhR is crucial for the manifestation of the observed dioxin-induced effects, and also impacts the normal bone phenotype as lack of AhR resulted in slightly modified bone tissue properties, both similar and opposite to effects of TCDD-exposure. Further, the outcome is clearly influenced by the timing of TCDD-exposure, as prenatal exposure resulted in delayed matrix maturation, while adult exposure caused a harder and stiffer bone matrix.

Based on the observations in the experimental models in this study, the overall results show that environmental contaminants, to which humans are continuously exposed, have the ability to modulate the osteogenesis process. The functional consequences of such modulations should be further elucidated in order to establish any causal links between exposure to everyday chemicals and effects on the bone tissue properties, and possible contribution to bone disorders.

# LIST OF PUBLICATIONS

I. **Herlin M**, Kalantari F*, Stern N*, Sand S, Larsson S, Viluksela M, Tuomisto JT, Tuomisto J, Tuukkanen J, Jämsä T, Lind PM, Håkansson H. Quantitative characterization of changes on bone geometry, mineral density and biomechanical properties in two rat strains with different Ah-receptor structures after long-term exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Toxicology, 2010; 273: 1-11*

II. Finnilä MAJ, Zioupos P, **Herlin M**, Miettinen HM, Simanainen U, Håkansson H, Tuukkanen J, Viluksela M, Jämsä T. Effects of 2,3,7,8-Tetrachlorodibenzo-p-dioxin exposure on bone material properties.
*Journal of Biomechanics, 2010; 43: 1097-1103.*

III. **Herlin M***, Finnilä MAJ*, Zioupos P, Aula A, Risteli J, Miettinen HM, Jämsä T, Tuukkanen J, Korkalainen M, Håkansson H, Viluksela M. New insight to the role of aryl hydrocarbon receptor in bone phenotype and in dioxin-induced modulation of bone microarchitecture and material properties.
*Manuscript*

IV. Elabbas LE*, **Herlin M***, Finnilä MA, Rendel F, Stern N, Trossvik C, Bowers WJ, Nakai J, Tuukkanen J, Viluksela M, Heimeier RA, Åkesson A, Håkansson H. In utero and lactational exposure to Aroclor 1254 affects bone geometry, mineral density and biomechanical properties of rat offspring.
*Toxicology Letters, 2011; 207: 82-88*

V. **Herlin M**, Korkalainen M, Ringblom J, Öberg M, Heimeier RA, Joseph B, Viluksela M, Håkansson H. The polybrominated biphenyl mixture Aroclor 1254 exhibits predominantly dioxin-like effects on osteoblast differentiation.
*Manuscript*

VI. **Herlin M**, Esteban J, Barber X, Heimeier RA, Korkalainen M, Joseph B, Viluksela M, Håkansson H. The role of retinoids in TCDD-induced bone tissue modulations: *in vivo* and *in vitro* study result evaluated by PLS.
*Manuscript*

* Authors contributed equally to this study

# CONTENTS

# LIST OF ABBREVIATIONS

| | |
|---|---|
| AhR | Aryl hydrocarbon receptor |
| AhRR | Aryl hydrocarbon receptor repressor |
| ALP | Alkaline phosphatase |
| ANOVA | Analysis of variance |
| ARNT | Aryl hydrocarbon receptor nuclear translocator |
| BMD | Benchmark dose |
| BMDL | Lower confidence bound on the benchmark dose |
| BMR | Benchmark dose response |
| CES | Critical effect size |
| CRABP | Cellular retinoic acid binding protein |
| CTX | Collagen I carboxy terminal telopeptide |
| CYP | Cytochrome P450 |
| DDE | 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane |
| EDCs | Endocrine disrupting compounds |
| HBCD | Hexabromocyclododekane |
| LO(A)EL | Lowest observed (adverse) effect level |
| MC | Metylcholantren |
| NO(A)EL | No observed (adverse) effect level |
| OCN | Osteocalcin |
| OPG | Osteoprotegerin |
| PAS | Per-ARNT-Sim |
| PCB | Polychlorinated biphenyl |
| PCDD | Polychlorinated dibenzo-p-dioxin |
| PCDF | Polychlorinated dibenzofurans |
| PINP | Procollagen I amino-terminal propeptide |
| PLS | Partial least square |
| pQCT | Peripheral quantitative computed tomography |
| RALDH | Retinaldehyde dehydrogenase |
| RANK | Receptor activator of nuclear factor κB |
| RANKL | Receptor activator of nuclear factor κB ligand |
| RAR | Retinoic acid receptor |
| REP | Relative potency |
| RUNX2 | Runt-related transcription factor 2 |
| RXR | Retinoid X receptor |
| TBT | Tributyltin |
| TCDD | 2,3,7,8-tetrachlorodibenzo-p-dioxin |
| TEF | Toxic equivalency factors |
| TEQ | Toxic equivalent |
| μ-CT | Micro-computed tomography |
| XRE | Xenobiotic response element |

# 1 INTRODUCTION

The industrialized society produces and handles numerous industrial chemicals, which are released into the natural environment. Many of these chemicals are so called persistent organic pollutants, which are long-lived compounds that can induce a broad range of pathological changes, affecting different organs and tissues. A variety of such compounds disrupts endocrine systems, and are usually referred to as endocrine disrupting compounds (EDCs). Originally EDCs were recognized to primarily cause reproductive effects. However, the group of compounds identified as EDCs has been found to be heterogeneous and to interact with various regulatory systems, affecting numerous signaling pathways, and new endpoints have been revealed as targets for EDCs. Bone, whose process for turnover and homeostasis is dependent on complex interactions of various cell types as well as of both local and systemic factors, such as hormones, growth factors and cytokines, is a potential target for the insult by chemicals with EDC properties. Emerging evidence has shown that exposure to compounds that bind to the aryl hydrocarbon receptor (AhR) can interfere with bone tissue. In the present thesis, bone tissue modulations following exposure to dioxin and dioxin-like compounds have been characterized, and the role of AhR for the observed effects was investigated. Quantitative approaches were used to evaluate both *in vivo* and *in vitro* endpoints, and possible associations with retinoid system disturbances in the manifestation of bone modulations were assessed.

## 1.1 DIOXINS AND DIOXIN-LIKE COMPOUNDS

Dioxins and related compounds are persistent organic pollutants that are ubiquitously present in the environment, resulting in continuous exposure of both humans and animals. These chemicals elicit a similar spectrum of toxicological responses through a common mechanism of action, but with variable potency. Reported health effects include reproductive impairment, developmental toxicity, immunotoxicity, neurotoxicity, hepatotoxicity, carcinogenesis, cardiovascular toxicity, skin toxicity, bone and tooth toxicity, and disruption of endocrine signaling systems, as well as a number of biochemical changes (White and Birnbaum 2009).

The most potent and toxic congener in the group of dioxins is 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), which is often used as a model substance for these types of compounds. In the ambient environment, dioxins and dioxin-like compounds are always present as mixtures, also containing non-dioxin-like compounds. The contribution of the non-dioxin-like congeners to the toxicity of such mixtures is largely unclear, as are their mechanisms of action. However, pattern of effects showing both differences and similarities with dioxin-like congeners have been demonstrated (Elabbas et al. 2013). As experimental studies have mostly focused on exposure to single compounds, not much is known about the possible implication on the effect pattern and adversity by the exposure to a combination of multiple environmental chemicals. For dioxin-like compounds, however, the system of so called toxic equivalency factors (TEFs) has been established in order to compare the potency of a compound, or a mixture of compounds, to produce toxic effects relative to that of TCDD (Table 1). For inclusion in the TEF concept, a compound must show a structural

relationship to the dioxins, be persistent and accumulate in the food chain, bind to the AhR and elicit AhR-mediated biochemical and toxic responses (Van den Berg et al. 2006). The group of dioxins and dioxin-like compounds includes polychlorinated dibenzo-p-dioxins (PCDDs) and polychlorinated dibenzofurans (PCDFs), which are by-products of certain industrial processes and combustion. Also some of the polychlorinated biphenyl (PCB) congeners, which constitute a group of industrial chemicals used in transformers and capacitors, are classified as dioxin-like due to their biological activity.

**Table 1.** Toxic equivalency factors for polychlorinated dibenzo-p-dioxins, polychlorinated dibenzofurans and dioxin-like polychlorinated biphenyls.

| Compound | TEF | Compound | TEF |
|---|---|---|---|
| **Polychlorinated dibenzo-p-dioxins** | | **Dioxin-like polychlorinated biphenyls** | |
| 2,3,7,8-TCDD | 1 | 3,3´4,4´-tetraCB (PCB 77) | 0.0001 |
| 1,2,3,7,8-PeCDD | 1 | 3,4,4´,5-tetraCB (PCB 81) | 0.0003 |
| 1,2,3,4,7,8-HxCDD | 0.1 | 3,3´4,4´5-pentaCB (PCB 126) | 0.1 |
| 1,2,3,6,7,8-HxCDD | 0.1 | 3,3´4,4´,5,5´-hexaCB (PCB 169) | 0.03 |
| 1,2,3,7,8,9-HxCDD | 0.1 | 2,3,3´4,4´-pentaCB (PCB 105) | 0.00003 |
| 1,2,3,4,6,7,8-HpCDD | 0.01 | 2,3,4,4´,5-pentaCB (PCB 114) | 0.00003 |
| OCDD | 0.0003 | 2,3´4,4´,5-pentaCB (PCB 118) | 0.00003 |
| **Polychlorinated dibenzofurans** | | 2´,3,4,4´,5-pentaCB (PCB 123) | 0.00003 |
| 2,3,7,8-TCDF | 0.1 | 2,3,3´,4,4´5-hexaCB (PCB 156) | 0.00003 |
| 1,2,3,7,8-PeCDF | 0.03 | 2,3,3´,4,4´,5´-hexaCB (PCB 157) | 0.00003 |
| 2,3,4,7,8-PeCDF | 0.3 | 2,3´,4,4´,5,5´-hexaCB (PCB 167) | 0.00003 |
| 1,2,3,4,7,8-HxCDF | 0.1 | 2,3,3´,4,4´,5,5´-heptaCB (PCB 189) | 0.00003 |
| 1,2,3,6,7,8-HxCDF | 0.1 | | |
| 1,2,3,7,8,9-HxCDF | 0.1 | | |
| 2,3,4,6,7,8-HxCDF | 0.1 | | |
| 1,2,3,4,6,7,8-HpCDF | 0.01 | | |
| 1,2,3,4,7,8,9-HpCDF | 0.01 | | |
| OCDF | 0.0003 | | |

### 1.1.1 The aryl hydrocarbon receptor

Most of the toxic effects of dioxins and dioxin-like compounds are mediated via activation of the AhR, a ligand-activated transcription factor belonging to the Per-ARNT-Sim (PAS) family of proteins (Hankinson 1995). The AhR is ubiquitously expressed in most organs and cells in the body, and besides its role in responses to toxic xenobiotic chemicals, it is also thought to play important roles in normal cell physiology. Mice lacking a functional AhR are largely resistant to the toxicity of TCDD, but also have a number of phenotypical abnormalities such as immune system impairment, liver fibrosis, heart hypertrophy and dermal fibrosis (Fernandez-Salguero et al. 1995; Gonzalez and Fernandez-Salguero 1998), providing strong evidence for a physiological role of the AhR. No definitive endogenous AhR-ligand has been identified, however, a number of candidates have been suggested, including arachidonic acid metabolites, heme metabolites, tryptophan metabolites and UV

photoproducts of tryptophan, as well as natural flavonoids and indole-3-carbinol derivatives (Denison and Nagy 2003; Nguyen and Bradfield 2008).

The AhR interacts with numerous other signaling pathways (reviewed in Puga et al. 2009), and it has been demonstrated that the presence or absence of AhR affects the expression of almost as many genes as TCDD-induced activation of the AhR, but with partly different expression pattern (Tijet et al. 2006). It is therefore likely that the toxic effects of exposure to potent and persistent AhR-ligands such as dioxins, are results of dysregulation of normal AhR activation, leading to inappropriate gene regulation and signaling in target cells (Bock and Kohle 2006). As illustrated in Figure 1, unliganded AhR resides in the cytosol, bound to a chaperon complex (rewieved in Petrulis and Perdew 2002). Ligand binding to the AhR induces translocation into the nucleus (Ikuta et al. 2000; Pollenz 1996) and release of the chaperons and heterodimerization of AhR with its partner protein aryl hydrocarbon receptor nuclear translocator (ARNT) (Heid et al. 2000; Reyes et al. 1992). In the nucleus, the AhR-ARNT heterodimer can bind to xenobiotic response elements (XREs) in the promoter region of target genes (reviewed in Swanson 2002) and regulate gene transcription. After transcription, AhR dissociates from ARNT and translocates in to the cytosol, where it is degraded (Davarinos and Pollenz 1999). The AhR activity can be regulated by various mechanisms. One such mechanism is negative feedback regulation by the AhR repressor (AhRR), whose expression is induced upon activation of the AhR (reviewed in Hahn et al. 2009). The exact mechanism by which the AhRR inhibits the AhR is not clear, but it has been hypothesized that it competes with AhR for heterodimerization with ARNT and binding to the XRE (Mimura et al. 1999).

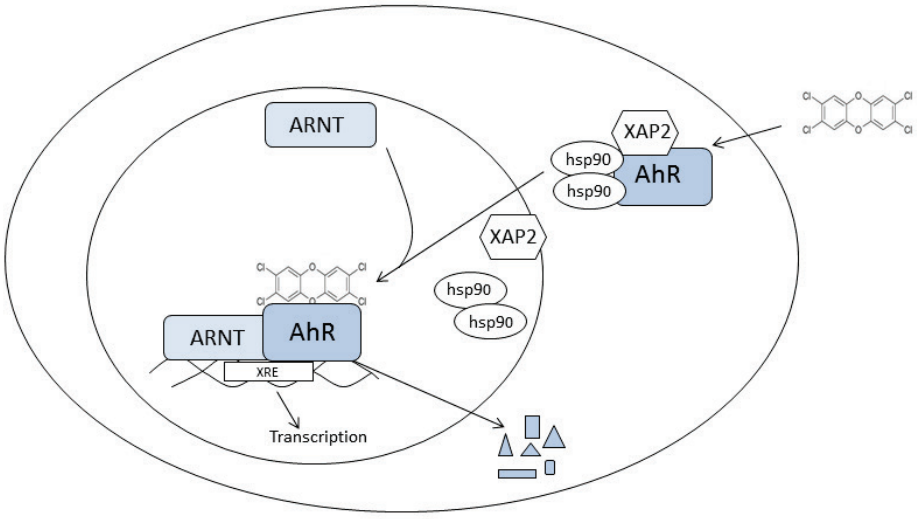


**Figure 1.** Illustration of the mechanisms behind ligand-activation of AhR-induced transcriptional regulation.

### 1.1.2 Interactions between the AhR and the retinoid system

The retinoid system is known to be a target in dioxin toxicity, and the disturbances of retinoid signaling is proposed to be involved in the effects of dioxins (Murphy et al. 2007; Nilsson and Hakansson 2002; Novak et al. 2008; Zile 1992). Alterations of the

retinoid system have therefore been suggested as an endpoint in the evaluation of chemicals with endocrine disrupting properties (OECD 2011). Retinoids play an essential role in the development and homeostasis of tissues, and both deficiency and excess have been associated with malformations (Zile 2001). Retinoids are non-steroid hormones that are obtained from the diet, and are present as several different forms, including retinyl esters, retinol, retinal and retinoic acids. In addition, a number of enzymes and binding proteins are associated with the retinoid metabolism and transport (Blomhoff and Blomhoff 2006). An overview of the retinoid metabolism and signaling in the cell is shown in Figure 2. Retinoids are mainly stored in the liver in the form of esters, and transported as retinol to provide tissues with optimal retinoid amounts (Blomhoff and Blomhoff 2006). In the cell, retinol is converted via retinal to *all-trans* retinoic acid, which is the signaling retinoid form. *All-trans* retinoic acid binds to retinoic acid receptors (RARs), thereby regulating the expression of retinoid target genes. *All-trans* retinoic acid can be further metabolized to generate retinoic acid isomers, of which some binds to RARs and/or retinoid X receptors (RXRs), while other are excreted.

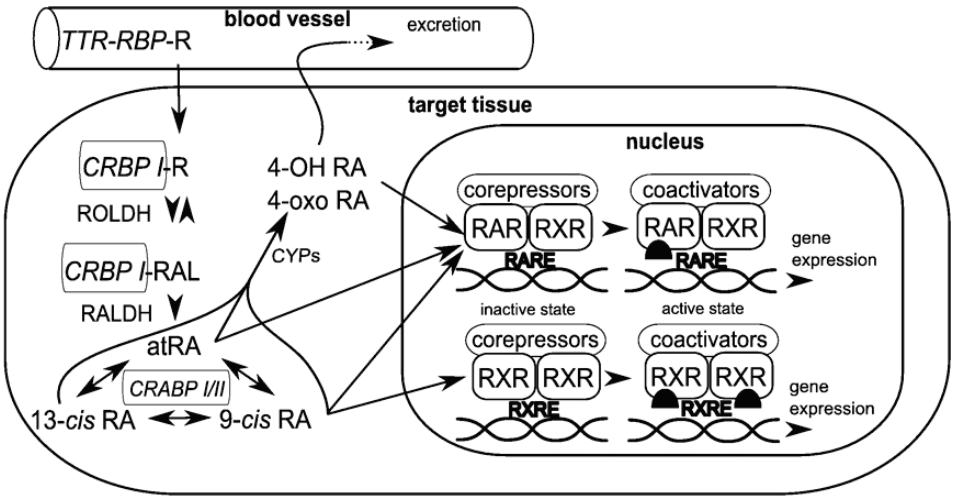


**Figure 2.** Retinoid metabolism and signaling in target cells. Reprinted from (Novak et al. 2008) with permission from Elsevier.
atRA: *all-trans* retinoic acid; CRBP: cellular retinol binding protein; CRABP: cellular retinoic acid binding protein; CYP: cytrochrome P450; R: retinol; RA: retinoic acid; RAL: retinal; RALDH: retinaldehyde dehydrogenase; RAR: retinoic acid receptor; RARE: retinoic acid response element; RBP: retinol-binding protein; RXR: retinoid X receptor; RXRE: retinoid X response element;TTR: transthyretin.

Exposure to dioxins and dioxin-like compounds induces tissue- and cell-specific changes in retinoid levels (reviewed in Nilsson and Hakansson 2002), and various interactions between the AhR- and retinoic acid-signaling pathways have been demonstrated (reviewed in Murphy et al. 2007). The mechanisms behind these observations are not well known, but it is likely that interactions between the AhR and retinoic acid pathways have the ability to modulate the synthesis, metabolism, storage and transport of retinoids, as well as transcriptional regulations (Murphy et al. 2007). AhR-knockout mice are shown to have reduced retinoic acid metabolism and elevated hepatic levels of retinoic acid, retinol and retinyl palmitate (Andreola et al. 1997), as well as altered retinoid and thyroxin metabolic response following TCDD-exposure (Nishimura et al. 2005). Modulations of the levels of signaling retinoids, which are

ligands to RARs and RXRs, are likely to alter the expression of retinoid-responsive genes, thereby having potential consequences for retinoid-regulated systems. Hundreds of genes have been suggested to be regulatory targets of retinoic acid (Balmer and Blomhoff 2002), indicating that a disturbed retinoid signaling could have the capacity to affect various systems, which is compatible with the profile of dioxin toxicity that includes a broad range of effects.

## 1.2 BONE TISSUE

Bone is a specialized form of connective tissue in which the extracellular matrix is mineralized. The bone tissue can be divided into cortical and trabecular (also called cancellous) bone. Cortical bone is compact and builds the outer shell of the bone, while trabecular bone is more sponge-like in structure, and is located inside of the cortical bone shell, in long bones mostly at the end sections. The micro-structure of cortical bone consists of cylindrical structures called osteons (Figure 3). The osteons are built of plate-like layers of bone matrix, the lamellae, which are wrapped around a central canal, the Haversian canal, containing nerves and blood supplies of the bone. The micro-structure of trabecular bone is also composed of lamellae, but which are arranged in an irregular network of rod- and plate-like elements, called trabeculae, instead of osteons. The lamellae of both osteons and trabeculae are built of mineralized collagen fibers, which consist of bone mineral crystals (hydroxyapatite, calcium carbonate, magnesium hydroxide, fluoride and sulfate), collagen molecules and other proteins (*e.g.* osteopontin, bone sialoprotein, osteocalcin and osteonectin) (Rho et al. 1998).

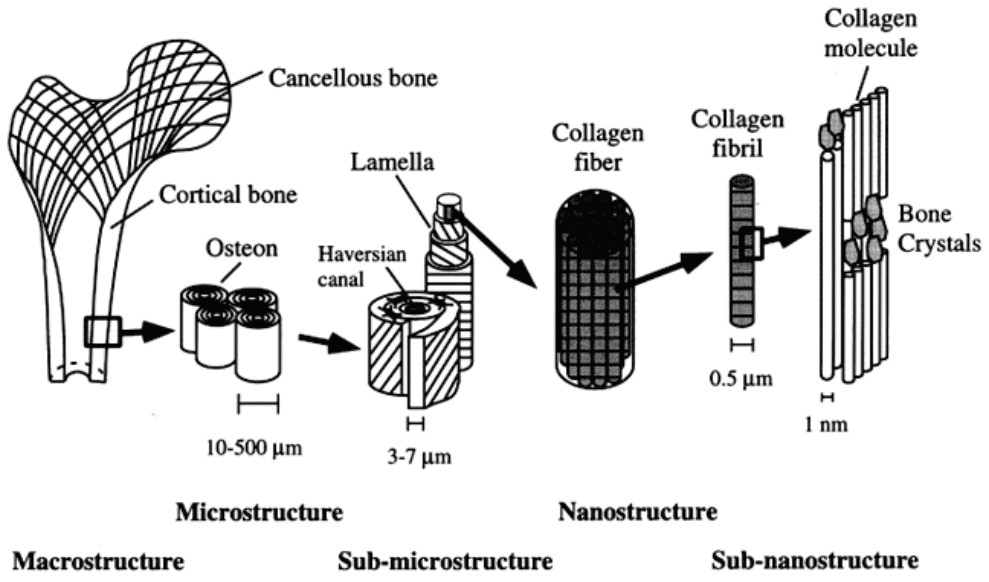


**Figure 3.** Hierarchical organization of bone. Reprinted from (Rho et al. 1998) with permission from Elsevier.

Bone strength is dependent on both geometrical, micro-structural and material properties of the bone. The bone tissue has to be stiff enough to resist deformation upon loading, but also flexible enough to be able to change in shape without breaking when load is applied. The collagen fibers in the matrix provide elasticity and flexibility, and the minerals that are crystallized onto the collagen fibers give the hardness of the bone (Burstein et al. 1975). If the degree of mineralization is too high, the stiffness increases but the flexibility decreases, making the bone more brittle. If the degree of mineralization is too low, the flexibility of the bone tissue increases while the stiffness decreases, resulting in the bone bending too much when load is applied.

Bone tissue is formed by endochondral or intramembranous ossification. Endochondral bone formation, which takes place in long bones, develops via a cartilaginous template that is remodeled into mineralized bone, while intramembranous bone formation, which takes place in flat bones, is formed without a cartilaginous template (Marks and Odgren 2002). Bone is a dynamic tissue that is continuously remodeled in order to renew the bone and repair microfractures, and to meet the mechanical needs. The bone tissue can adapt in terms of changes in mineral density, in disposition of trabeculae and osteons, and in terms of shape and dimensions (Turner 1998). A schematic overview of bone cell types involved in bone remodeling is shown in Figure 4. Osteoclasts are multinucleated cells, derived from hematopoietic stem cells, which resorb bone by acidification and proteolysis of the bone matrix. Osteoblasts are derived from mesenchymal stem cells, and work in clusters during the formation of new bone, which occurs by deposition of collagen followed by mineralization of the matrix. Osteocytes, which are the most abundant cell type in mammalian bone, are mature osteoblasts that at the end of the bone formation phase have become embedded in the matrix. Osteocytes form an interconnected network of cells that are thought to have the capacity to sense mechanical loads and detect microfractures in the bone, and respond by activating osteoclasts (Cullinane 2002; Heino et al. 2009; Xiong and O'Brien 2012). Also lining cells, which are covering the surface of mineralized bone matrix, originate from osteoblasts and are suggested to be involved in controlling the microenvironment and signals regulating bone remodeling (Miller et al. 1989).

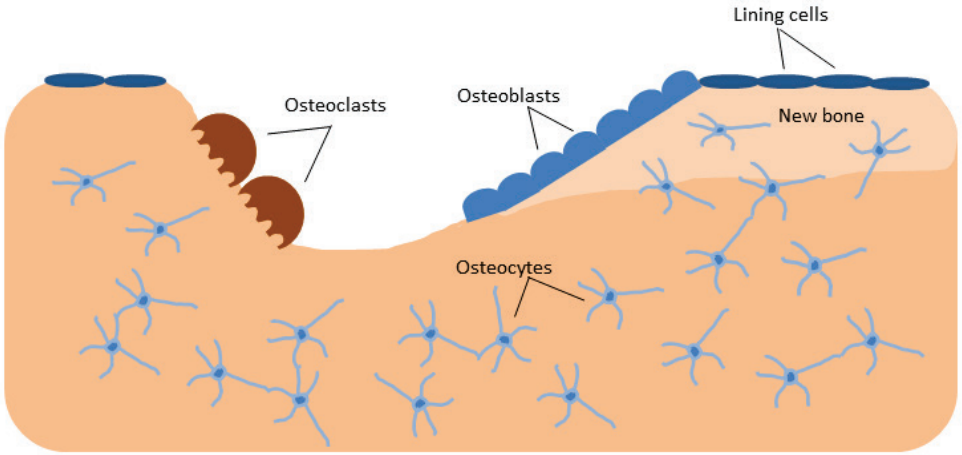


**Figure 4**. Illustration of cell types involved in the bone remodeling process.

Bone remodeling is regulated by both systemic and local factors, such as hormones and cytokines. One central regulatory system is the OPG/RANKL/RANK system that constitutes a direct interaction between the osteoblast-linage cells and osteoclasts (Hofbauer et al. 2000; Xiong and O'Brien 2012). The osteoblasts express the receptor activator of nuclear factor κB ligand (RANKL), which binds to the receptor activator of nuclear factor κB (RANK) on osteoclast precursors and stimulate differentiation of osteoclasts. Osteoblasts also produce osteoprotegerin (OPG) that blocks the effects of RANKL. This system thereby ensures that the processes of resorption and formation of bone are tightly coupled and thus in equilibrium.

## 1.3 DIOXIN-RELATED BONE TISSUE ALTERATIONS

An increasing number of studies suggest that bone tissue should be considered as a target in environmental toxicology. It has been observed that exposure to dioxins and dioxin-like compounds has the ability to modulate bone tissue in terms of altered bone geometry, bone mineral density and bone strength. Jämsä et al. (2001) studied bone effects in adult rats of two strains with different AhR-structures, following long-term exposure to TCDD, and demonstrated decreased cross-sectional and medullary areas, as well as reduced mechanical strength of tibial diaphysis (Jamsa et al. 2001). Further, the difference in sensitivity to dioxin-induced bone alterations between the two rat strains indicated that the observed effects on bone are AhR-dependent (Jamsa et al. 2001). Miettinen et al. (2005) studied the effects of TCDD on developing rat bone following maternal exposure at different times of gestation and lactation, and showed decreased cross-sectional area and bone mineral density, as well as reduced mechanical strength of tibia, femur and femoral neck. The effects were found to be dependent on the timing of exposure, as earlier exposure caused more pronounced effects, while at the age of one year, along with discontinued exposure, most of the effects were reversed (Miettinen et al. 2005). Consistent with the findings by Jamsa et al. (2001), the TCDD-induced bone effects were observed mainly in offspring of the rat strain with a wild-type AhR, and less in rats with an altered receptor type (Miettinen et al. 2005). Decreased tibial trabecular area, increased bone mineral density and alteration of the chemical composition of bone were shown following short-term TCDD-exposure of adult rats (Lind et al. 2009b), and mice exposed to TCDD via lactation showed reduction in mineralized bone in the proximal end of tibia (Nishimura et al. 2009). Bone effects of TCDD-exposure have also been shown for other species. Hermsen et al. (2008) reported increased cross-sectional bone area and bone mineral content in female rhesus monkey, and altered macro-mechanical properties in the males, following in-utero and lactational TCDD-exposure (Hermsen et al. 2008). Studies in fish have shown malformations of craniofacial skeletal structures and underdeveloped ribs in rainbow trout sac-fry (Hornung et al. 1999), malformation of bone in medaka (Kawamura and Yamashita 2002), and disrupted bone growth in medaka embryos (Dong et al. 2012), following exposure to TCDD.

In addition to TCDD, also other dioxin-like compounds have been demonstrated to affect bone. Exposure of rats to PCB126, the most dioxin-like PCB congener, has been shown to result in increased cortical thickness (Lind et al. 2004; Lind et al. 1999), higher organic bone content and larger osteoid surface (Lind et al. 1999), and decreased mechanical strength and changes in the composition of the organic matrix (Lind et al.

2000) of long bones. Further, decreased degree of mineralization, altered bone mineral composition and increased trabecular bone mineral density has been demonstrated in rat vertebrae following exposure to PCB126 (Alvarez-Lloret et al. 2009). Exposure of nestling american kestrels to PCB126 has been shown to result in decreased skeletal growth (Hoffman et al. 1996), and chicks from PCB-exposed hens showed deformities of legs, feet, and skulls (Summer et al. 1996). Fetuses of ewes exposed to PCB153 were shown to have decreased cross-sectional bone area, thicker cortical bone, decreased marrow cavity and increased bone mineral density of femurs (Gutleb et al. 2010). Further, sheep exposed to PCBs, among other contaminants, through contaminated pastures, showed alterations in femoral bone areas and mineral density (Lind et al. 2009a; Lind et al. 2010). Both skull bone and long bone of terrapins were affected by exposure to PCB126, resulting in altered geometrical and densitometrical properties (Holliday and Holliday 2012), and frogs exposed to a metabolite of 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane (DDE) showed decreased cortical bone mineral density (Lundberg et al. 2007). Exposure to Aroclor 1254, a PCB mixture containing both dioxin-like and non-dioxin-like congeners, was shown to result in decreased cross-sectional and cortical bone areas, and proportionally smaller medullary area, as well as increased bone mineral density and reduced bone strength of long bone in rats (Andrews 1989). Also decreased collagen content in long bones (Ramajayam et al. 2007) and alterations of cortical bone structure in vertebrae (Yilmaz et al. 2006) has been reported following exposure of rats to Aroclor 1254.

Perinatal exposure to the AhR-ligand 3-metylcholatren (3MC) resulted in abnormalities and delayed ossification of metacarpals and metatarsals in mouse foetuses (Naruse et al. 2002). Further, the CA-AhR mouse, which has a constitutively active AhR and is used as a model for effects of continuous low-level activation of the AhR (Brunnberg et al. 2006), has been observed to have a bone phenotype differing from wild-type mice, such as higher cross-sectional and trabecular bone areas, higher trabecular bone mineral content, and macro-mechanical properties indicative of less brittle bone (Wejheden et al. 2010).

A number of wild-life studies have found correlations between exposure to organochlorine mixtures, which usually contains dioxins and/or dioxin-like compounds, with alterations of bone tissue. Grey seals from the Baltic Sea sampled during the period of high organochlorine contamination showed bone loss in skulls (Bergman et al. 1992) and lower trabecular bone density of radius (Lind et al. 2003), compared to seals collected during periods with lower contamination. Reduced bone mineral density of skulls has been associated with high concentrations of organochorines, including PCBs, also in polar bears (Sonne et al. 2004). In otters, elevated muscle concentrations of PCBs have been related to increased cortical area, cortical thickness and cortical bone mineral content (Roos et al. 2010). Alligators from a pesticide contaminated lake showed increased bone mineral density (Lind et al. 2004), and bank voles from a dioxin contaminated area were shown to have reduced strength of femural neck (Murtomaa et al. 2007). Clapper rail chicks from a site contaminated with PCBs, among other toxicants, showed altered chemical composition of the bone (Rodriguez-Navarro et al. 2006), and skeletal deformities in grey heron chicks have been linked to organochlorine contamination (Thompson et al. 2006). Herring gulls from organochlorine contaminated areas had shorter and thinner femoral

bone with lower mineral density (Fox et al. 2008). Fish exposed to pulp mill effluents, containing organochlorines, have shown vertebral deformities and altered mechanical properties of vertebrae (Bengtsson et al. 1988).

Only a few epidemiological studies have assessed correlations between dioxin-related exposure and changes of bone tissue properties in humans. There are studies that give support for a high dietary intake of organochlorines as a risk factor for vertebral fractures (Alveblom et al. 2003), and indications that exposure to organochlorine affects bone mineral density (Hodgson et al. 2008). But there are also studies that provide minor or no support for an association between organochlorine exposure and effects on bone mineral density (Cote et al. 2006; Glynn et al. 2000) or risk of osteoporotic fractures (Wallin et al. 2004). Further, levels of DDE in serum has been suggested to correlate with reduced bone mineral density in women (Beard et al. 2000), while another study showed no correlation between DDE-exposure and bone mineral density (Bohannon et al. 2000).

*In vitro* studies have reported dioxin-induced effects on both osteoblasts and osteoclasts. Modulation of osteoblast differentiation was demonstrated following exposure to TCDD (Carpi et al. 2009; Gierthy et al. 1994; Korkalainen et al. 2009; Koskela et al. 2012; Ryan et al. 2007; Singh et al. 2000), Aroclor 1254 (An et al. 2012), as well as 3MC (Naruse et al. 2002). Exposure to 3MC was shown to also affect the differentiation and fusion, but not the resorption activity, of osteoclasts (Naruse et al. 2004). Consistently, TCDD-exposure of osteoclasts had no effect on the number or activity of osteoclasts (Ilvesaro et al. 2005), but affected osteoclast differentiation (Korkalainen et al. 2009; Koskela et al. 2012). Also benzo[a]pyrene has been shown to affect osteoclastogenesis (Voronov et al. 2005; Voronov et al. 2008). Expression of the AhR has been demonstrated both in histological bone sections (Ilvesaro et al. 2005), and in osteoblasts and osteoclasts *in vitro* (Ilvesaro et al. 2005; Korkalainen et al. 2009; Naruse et al. 2002). Further, the AhR-antagonist resveratrol has been shown to inhibit TCDD-induced effects on osteoblasts (Singh et al. 2000), and consistently, osteoblastic cells from AhR-knockout mice were unaffected by TCDD (Korkalainen et al. 2009), suggesting the effects to be mediated through the AhR. Resveratrol also partially inhibited the effects of 3MC (Naruse et al. 2004) and benzo[a]pyrene (Voronov et al. 2005) on osteoclastic cells.

In spite of the associations between dioxin exposure and modulations of bone, both *in vivo* and *in vitro*, the mechanisms behind the observed effects are not well understood. As signaling systems are sensitive to chemical interference, the regulation of bone tissue homeostasis is a potential target for any disturbances that might cause imbalance in the regulation of bone modeling and remodeling. An adequate retinoid status is important for bone homeostasis, and effects on bone by retinoid excess have been demonstrated both experimentally *in vivo* and *in vitro*, and in humans (reviewed in Conaway and Lerner 2011). Both osteoblasts and osteoclasts express receptors for retinoic acid (Kindmark et al. 1993; Saneshige et al. 1995), and the significance of retinoid signaling in skeletogenesis is established (Weston et al. 2003). In humans, both too high (Feskanich et al. 2002; Melhus et al. 1998; Michaelsson et al. 2003; Opotowsky and Bilezikian 2004; Promislow et al. 2002) and too low (Opotowsky and Bilezikian 2004) retinoid intake has been associated with reduced bone strength. In

rodents, retinoid excess has been reported to result in altered bone properties, such as bone geometry and strength (Dhem and Goret-Nicaise 1984; Hough et al. 1988; Johansson et al. 2002; Kneissel et al. 2005; Lind et al. 2011). Moreover, it has been demonstrated that knock-out of the retinaldehyde dehydrogenase (RALDH3), which synthesize retinoic acid, blocks TCDD-induced cleft palate in mice, suggesting that retinoid signaling is necessary for TCDD to induce alteration of palate development (Jacobs et al. 2011). Further, in TCDD-exposed rats, serum levels of retinoids have been shown to be altered (Fletcher et al. 2005) at the same doses where effects on bone properties, especially altered bone geometry, have been observed (Jamsa et al. 2001). These findings suggest that the retinoid system may have a role in mediating bone toxicity by AhR-ligands.

# 2 AIMS OF THE PRESENT STUDY

The general objective of this thesis was to in detail characterize the effects of dioxins on bone tissue, and to investigate the role of AhR and retinoid system disturbances for the observed effects.

The specific aims were to:

- Characterize the effects on bone geometry, mineral density, micro-structure and bone material and macro-mechanical properties following adult, as well as perinatal, exposure to TCDD (**papers I, II, III**).

- Investigate the role of a functional AhR for TCDD-induced bone modulations (**papers I, III**), and for normal bone phenotype (**paper III**).

- Study bone modulations following exposure to a mixture of dioxin-like and non-dioxin-like PCB-congeners, and elucidate qualitative and quantitative similarities to the effects of TCDD (**papers IV, V**).

- Investigate the role of TCDD-induced retinoid system disturbances in relation to effects of bone tissue properties and osteoblast differentiation (**paper VI**).

- Apply the benchmark dose methodology in the evaluation of bone parameters to derive effects doses and relative potency values (**papers I, IV, V**).

# 3 COMMENTS ON METHODOLOGY

## 3.1 PERIPHERAL QUANTITATIVE COMPUTED TOMOGRAPHY

Peripheral quantitative computed tomography (pQCT) is an X-ray based technique used for assessment of geometrical and densitometrical properties of bone. The method has the ability to differentiate between cortical and trabecular bone tissue, and generates volumetric information from cross-sections of the bone. Examples of images from pQCT analyses are shown in Figure 5.

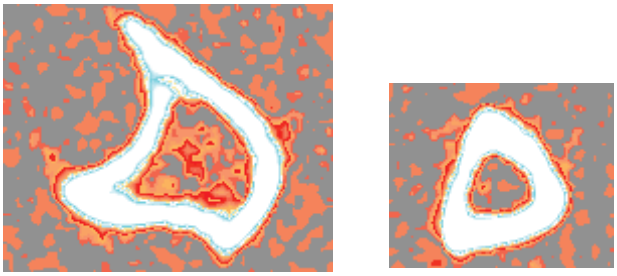

**Figure 5**. Examples of pQCT images at different sites of tibial bone.

By pQCT, the geometry of different bone compartment, as well as the bone mineral content and mineral density, are calculated. Also approximations of the macro-mechanical properties of the bone are provided by using the cross-sectional information about the bone sample (Gasser 1995). The pQCT scans generate a number of parameters, of which the most commonly used ones are listed in Table 2.

**Table 2**. Parameters generated by pQCT analyses.

| Parameter | Description |
|---|---|
| Total bone mineral content | The mineral content of the bone within a bone slice |
| Cortical bone mineral content | The mineral content of cortical bone within a bone slice |
| Trabecular bone mineral content | The mineral content of trabecular bone within a bone slice |
| Total bone mineral density | The mean volumetric density of the bone |
| Cortical bone mineral density | The mean volumetric density of the cortical bone |
| Trabecular bone mineral density | The mean volumetric density of the trabecular bone |
| Total bone area | The cross-sectional area of the bone |
| Cortical bone mineral area | The area of the cortical bone |
| Trabecular bone mineral area | The area of the trabecular bone |
| Periosteal circumference | The outer bone circumference |
| Endosteal circumference | The inner circumference of the cortical bone |
| Cortical thickness | The thickness of the cortical shell |
| Polar moment of inertia | Indication of the resistance to rotation of the bone |

## 3.2 MICRO-COMPUTED TOMOGRAPHY

Micro-computed tomography (μ-CT) is, similarly to pQCT, an X-ray based method for evaluation of bone properties. Compared to pQCT the μ-CT has a higher resolution, which gives more detailed information at the level of bone micro-structure. Examples of images from μ-CT analyses are shown in Figure 6.

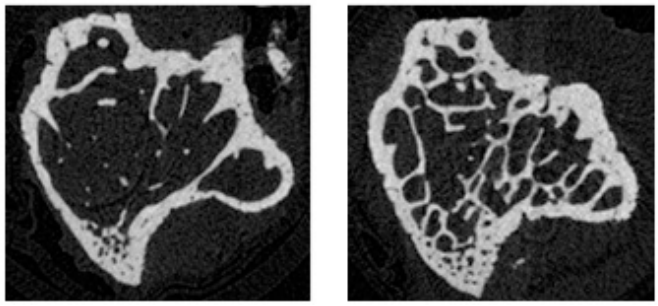

**Figure 6**. μCT images of tibial bone showing various numbers of trabeculae.

The higher resolution also results in less so called partial volume effect, which in pQCT-analyzes might affect the estimations of densitometrical values (Feretti 1999). As with the pQCT, a number of parameters are generated by μ-CT analyses, of which the most commonly used ones are listed in Table 3.

**Table 3**. Parameters generated by μ-CT analyses.

| Parameter | Description |
|---|---|
| Bone volume fraction | The fraction of a given volume that is occupied by bone |
| Trabecular thickness | Thickness of individual trabeculae |
| Trabecular separation | Thickness of the spaces between trabeculae |
| Trabecular number | Number of trabeculae in a bone region |
| Trabecular bone pattern factor | Indication of connectivity of the trabeculae |
| Structural model index | Indication of the relative prevalence of rod- and plate-like trabeculae |
| Porosity | The proportion of the cortical area that consist of enclosed spaces |
| Polar moment of inertia | Indication of the resistance to rotation of the bone |

## 3.3 THREE-POINT BENDING TEST

In order to evaluate the mechanical strength of bone, beyond the estimations by pQCT and μ-CT, biomechanical analyses are used. Three-point bending test (Figure 7A) is a method to analyze the bending strength of long bones (Leppanen et al. 2006). When a force is applied, the bone will start to deform and an internal resistance to the applied force is generated. The change in dimension is plotted against the load in a stress-strain curve (Figure 7B), which gives information about the macro-mechanical properties of the bone.

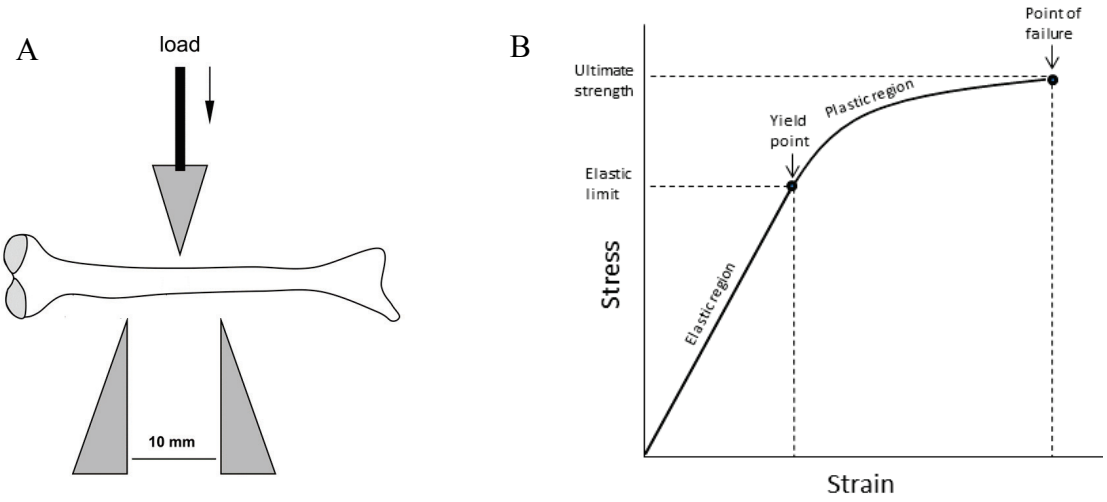


**Figure 7**. A) Three-point bending test (sketch by N Stern). B) Strain-stress curve

The linear part of the curve is the elastic region. During this stage of the bending, there are no permanent changes of the bone shape, and the bone is able to return to its original position if the loading is stopped. The slope of the elastic region gives the stiffness of the bone. If the load increases the curve will become non-linear, which is the plastic region. While in the plastic region, the bone is being permanently deformed by the load, and ultimately it will break. The main parameters received from the stress-strain curve in three-point bending test are listed in Table 4.

**Table 4.** Parameters generated by three-point bending test.

| Parameter | Description |
|---|---|
| Maximum breaking force | The force at which the bone breaks |
| Deformation | Deformation at the point of maximum breaking force |
| Stiffness | Stiffness of the bone |
| Energy absorption | The energy the bone is able to store until it brakes |

## 3.4 NANOINDENTATION

Nanoindentation is a technique that can be used to evaluate mechanical properties of bone matrix. Such nano-mechanical bone parameters give information of the matrix material properties. In this approach the cortical and trabecular bone are evaluated separately. A very fine diamond tip is loaded to the surface of the bone sample, and the loading and unloading of the tip is recorded (Ozcivici et al. 2008). An image of indentation sites in cortical bone is shown in Figure 8A. A load-displacement curve is obtained (Figure 8B), and the parameters are derived from the response of the material.

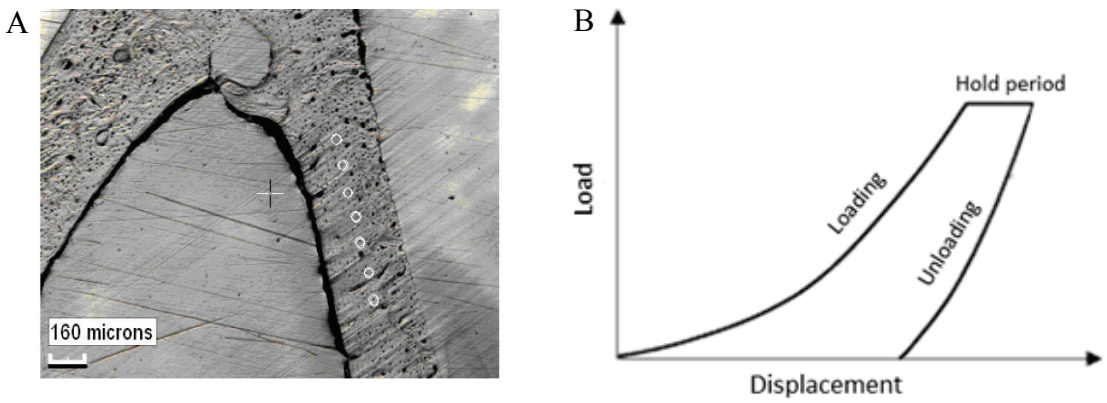


**Figure 8**. A) Indents in cortical bone. Reprinted from (Finnila et al. 2010), with permission from Elsevier. B) Load-displacement curve.

Both dynamic and quasistatic loading can be applied, allowing for different nano-mechanical properties to be evaluated. Elastic modulus is calculated from the slope of the elastic region of the unloading curve, and the hardness is given by the displacement at the maximal load. When the load is increased, the material is undergoing both elastic and plastic deformation. If the load is held constant (hold period), a time-dependent deformation is seen, the so called creep behavior, which describes the structural damage to the material from loading. The main parameters received from the load-displacement curve in nanoindentation tests are listed in Table 5.

**Table 5**. Parameters generated by nanoindentation test.

| Parameter | Description |
|---|---|
| Indentation hardness | Resistance to permanent deformation due to a constant load |
| Elastic modulus | Describes the elasticity on the material |
| Storage modulus | Describes the elastic portion of viscoelastic materials |
| Loss modulus | Describes the viscous portion of viscoelastic materials |
| Plasticity index | The ability to permanently change in shape without breaking |
| Creep amplitude | Describes the structural damage to the material from loading |

## 3.5 CELL LINE AND MARKERS OF OSTEOBLASTIC DIFFERENTIATION

The murine calvarial osteoprogenitor cell line MC3T3-E1 was used to study effects on osteoblast differentiation. Osteoblasts differentiate from progenitor cells into proliferating pre-osteoblasts, and further into bone matrix-producing osteoblasts. The differentiation of osteoblasts occurs in three principal phases, which are proliferation, extracellular matrix maturation and mineralization (Lian and Stein 1995). During these phases there are temporal expressions of differentiation-related genes that can be used as markers for osteoblast differentiation. MC3T3-E1 cells are shown to display such sequential expression analogous to *in vivo* bone formation, therefore providing a useful model for studying osteoblast differentiation (Quarles et al. 1992). In the present study, the mRNA expression of runt-related transcription factor 2 (RUNX2), alkaline phosphatase (ALP) and osteocalcin (OCN) were used (Figure 9). RUNX2 is a

transcription factor that is expressed during proliferation, ALP is expressed prior to the initiation of mineralization, and OCN is expressed in differentiated osteoblasts (Ducy 2000; Lian and Stein 1995).

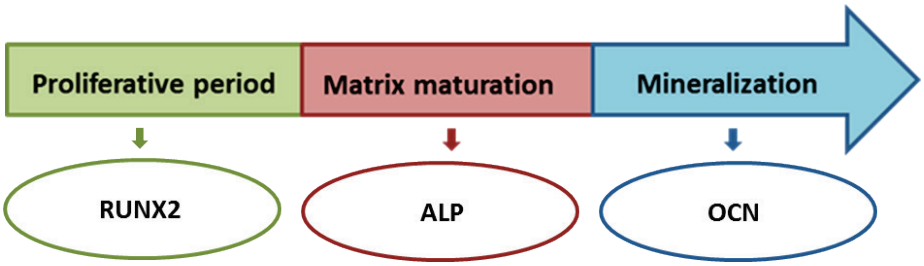


**Figure 9.** Principal phases of osteoblast differentiation and corresponding markers.
RUNX2: runt-related transcription factor 2; ALP: alkaline phosphatase; OCN: osteocalcin.

## 3.6 BENCHMARK DOSE MODELING

The benchmark dose (BMD) approach has been suggested as an alternative to the derivation of the "No Observed Adverse Effect Level" (NOAEL) or "Lowest Observed Adverse Effect Level" (LOAEL) in health risk assessment of chemicals. NOAEL is defined as the highest dose which does not cause statistically significant effects, and the LOAEL as the lowest dose which cause effects. In comparison to the NOAEL/LOAEL approach, the BMD method makes more use of the dose-response data and the BMD is not limited to be one of the experimental doses, which makes the results less dependent on the study design in terms of experimental dose levels and dose spacing.

To derive BMDs, a dose-response model is fitted to the experimental data, and the BMD corresponding to a certain level of response is estimated (Slob 2002) (Figure 10). This level of response, called critical effect size (CES), or benchmark dose response (BMR), is for continuous data typically a pre-defined percentage change relative to the background level of the particular endpoint (Sand et al. 2008; Slob 2002).

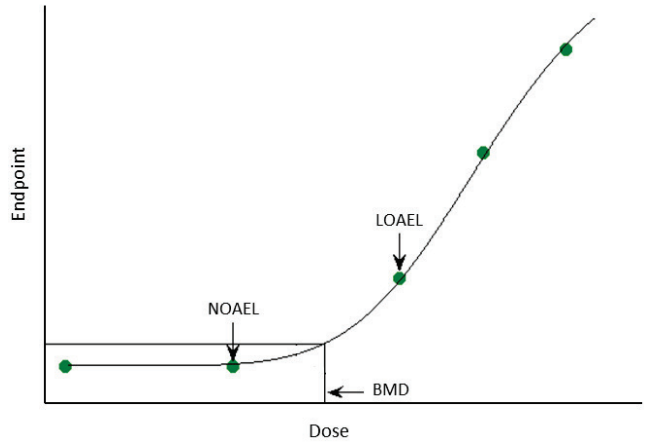


**Figure 10**. Schematic dose-response curve. A BMD is indicated in comparison to NOAEL and LOAEL.

The CES should represent a non-adverse but biologically relevant change in the response for a certain endpoint, and EFSA has recommended a CES of 5% as a default starting value (EFSA 2009). However, for some endpoints this effect level might not be relevant from a biological point of view, and especially for *in vitro* endpoints, such as effects on enzymes or gene expression, it is difficult to define a biologically appropriate CES. Also for endpoints not traditionally used in toxicological studies, where there is little knowledge about the consequences and adversity of the effect, a consensus regarding useful effect levels is lacking.

## 3.7 PARTIAL LEAST SQUARE

Partial least square (PLS) analysis is a multivariate regression method that is used to model the relationships between explanatory variables and response variables (Wold et al. 2001). PLS analyzes allows for geometrically visualization of the relations between the explanatory and response variables that are projected in two- (or more) dimensions, and thus associations between variables are ascertained.

# 4 RESULTS AND DISCUSSION

## 4.1 EFFECTS OF DIOXIN ON BONE TISSUE

**Long bones are suitable for the evaluation of effects on geometry, mineral density and macro-mechanical properties in studies of dioxin-induced bone toxicity**
In **paper I**, the effects of exposure of adult rats to TCDD were analyzed at different bone sites; long bones (femur and tibia), femoral neck and lumbar vertebra. The results show that in particular the cross-sectional geometry of long bones was a sensitive and consistently affected endpoint of TCDD-exposure of adult rats. Both periosteal and endocortical circumferences of tibia and femur were decreased, resulting in reduced cross-sectional, cortical and trabecular areas. Further, exposure to TCDD decreased trabecular bone mineral density of femur, and the lower maximal breaking force, stiffness and energy absorption of long bones indicated less stiff bones with lower ability to absorb energy. Such weakness would result in an increased risk of fracture at lower load.

The results from **paper I** suggest that analyses of long bones are more suitable for evaluation of effects on geometry, mineral density and macro-mechanical properties of bone in studies of dioxin-induced bone toxicity, than are femoral neck and lumbar vertebra. This finding does not necessarily reflect that long bones are more sensitive to TCDD-induced effects, but could be due to methodological reason, such as measuring reproducibility. However, it has been shown that the tibial and femural metaphysis reacts with the greatest magnitude of change to interventions such as ovariectomy (Gasser 1995), which may be true also for effects of dioxins.

**TCDD-exposure during development affects the bone matrix maturation process**
The exposure to environmental chemicals is of special concern for fetuses and infants as it occurs during the most vulnerable time-points of organ system development. In **paper II**, the effects of perinatal TCDD-exposure on bone tissue was evaluated for rat offspring at post-natal days (PND) 35 and 70. In particular, the material properties of cortical bone were studied using nanoindentation. The nano-mechanical parameter storage modulus describes the elasticity and stiffness of a material, hardness is a measure of the resistance to permanent deformation due to the load, and plasticity index reflects the ability to permanently change shape in response to the force without breaking. During normal bone matrix maturation, storage modulus and hardness is increased along with increase of the bone mineral content, while plasticity index decreases (Zioupos 2005; Zioupos and Currey 1998). When these parameters were compared between the offspring at PND 35 and PND 70 in **paper II**, the normal pattern was seen for the unexposed rats as the modulus and hardness was higher at PND 70 than at PND 35, and the plasticity index was lower. However, the TCDD-exposed rats did not show significant changes in these parameters between PND 35 and PND 70 (Figure 11), indicating that exposure to TCDD during development delays the normal bone matrix maturation process.

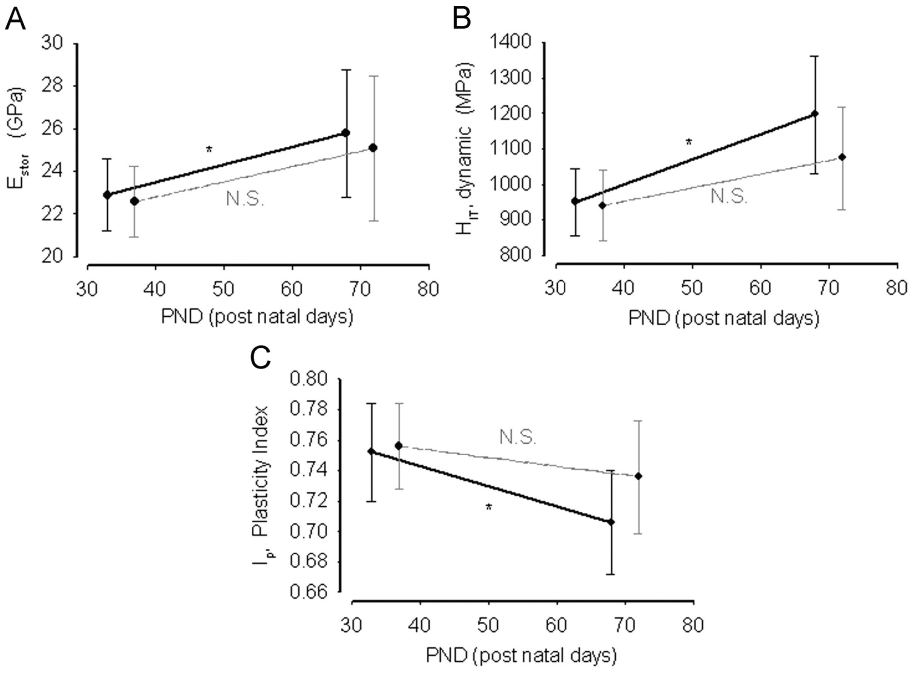


**Figure 11**. Nano-mechanical bone parameters A) modulus, B) hardness, and C) plasticity index in control (full line) and TCDD-exposed (dashed line) rat offspring at PNDs 35 and 70. Reprinted from (Finnila et al. 2010), with permission from Elsevier.

In addition to affecting bone material properties, TCDD-exposure also decreased the cross-sectional area and the cortical thickness, as well as reduced the cortical bone mineral density. The cross-sectional moment of inertia (reflecting the resistance to rotation) and moment of resistance (reflecting the resistance to bending) was decreased, as were also the bending force and stiffness of the bone. These results suggest that prenatal exposure to TCDD led to softer and more ductile bone, as indicated by less increase of the storage modulus and hardness, and less decrease of plasticity.

The mineral content of the bone is contributing to the major part of the elastic stiffness of the bone, whereas the plastic properties depend on the collagen (Burstein et al. 1975). The lower storage modulus, which is related to the mineral component of the bone, indicates a lower mineral-to-collagen ratio. Also the decreased bending force and stiffness is in agreement with lower mineralized bone in relation to the collagen content. When correlations between the affected parameters were analyzed, the bending force and stiffness showed stronger correlations with the mineralization and geometrical parameters, such as cortical bone mineral density and cortical area, than with the nano-mechanical parameters. The mechanical strength of whole bone depends on both geometry and bone material properties, and the results in this study indicate that the reduced bone strength observed in TCDD-exposed offspring was mainly a consequence of the altered bone geometry and mineralization level, rather than of changes in the bone material properties.

**TCDD-exposure alters the micro-structure and material properties of bone**

Bone tissue can be considered at multiple hierarchical levels, as illustrated in Figure 3, and bone quality is determined by structural as well as material properties. However, not much is known about the effects of dioxin toxicity on bone tissue at the level of micro-structure. In **paper III**, the effects of exposure of adult mice to TCDD were analyzed by μ-CT and nanoindentation, in addition to pQCT and three-point bending test. TCDD-exposure decreased the endosteal circumference of metaphysis, while the periosteal circumference was unaffected, resulting in a smaller trabecular area but unaffected cross-sectional area. Normally, the bone grows through resorption at the endosteal surface and lying down of bone on the periosteal surface, resulting in the bone becoming larger with a larger medullary area (Jaworski 1981), however, a smaller medullary or trabecular area have been observed also in other studies following exposure to TCDD (Herlin et al. 2010; Jamsa et al. 2001; Lind et al. 2009b) or PCBs (Andrews 1989). In spite of the smaller trabecular area, the trabecular bone volume fraction was increased, due to an increased number of trabeculae (Figure 12). This was also reflected by increased trabecular bone mineral density, which most likely is due to the increased trabecular number and not to bone mineral density increase in individual trabeculae. The difficulties to distinguish between a "real" increase in mineral density and increased number of trabeculae, using pQCT, are due to the voxel size of the pQCT that is of the magnitude of the trabecular thickness. Consequently, the voxels consist only partially of the bone tissue while the rest is bone marrow, resulting in so called partial volume effect (Feretti 1999). When, as in this study, the trabecular bone volume fraction is increased, there is more bone tissue in a given volume, which increases the apparent bone mineral density.

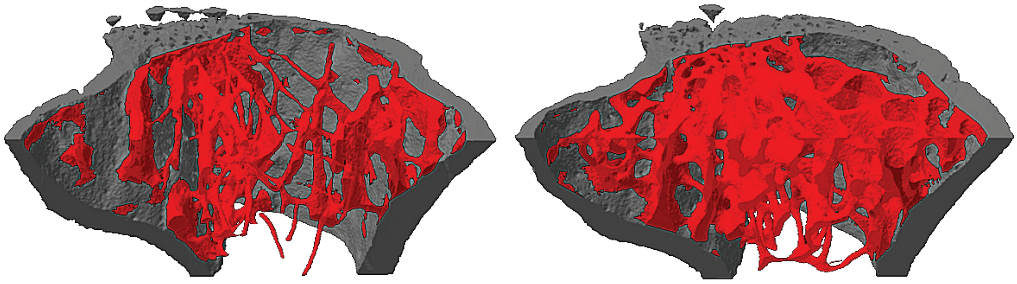

**Figure 12**. Bone tissue of unexposed (left) and TCDD-exposed (right) mice in paper III, illustrating the difference in trabecular bone volume fraction (image by M Finnilä).

Although the mineral density of the trabeculae themselves did not seem to be altered, the hardness and elastic modulus were increased, which indicated that the trabecular bone matrix became harder and stiffer. Further, the structural model index of trabecular bone was decreased by TCDD-exposure, indicating a more plate-like shape of the trabeculae. The finding of the trabeculae being more plate-like in shape is unlike the osteoporotic bone phenotype, for which the trabeculae are characterized by a more rod-like structure (Wehrli et al. 2001). The cortical bone was affected in terms of decreased thickness and increased porosity, and similarly to the trabecular bone matrix, the hardness and elastic modulus were increased by TCDD-exposure, indicating a harder cortical bone matrix.

As bone remodeling involves both removal of mineralized bone and formation of new bone matrix that becomes mineralized, imbalances in any of these processes affect the bone material properties. TCDD-exposure decreased the ratio of the bone formation marker procollagen I amino-terminal propeptide (PINP) and the bone resorption marker collagen I carboxy terminal telopeptide (CTX) in serum, indicating unbalanced bone remodeling. Consistent with these results, Lind et al. (2009) reported decreased PINP levels and increased CTX levels in combination with a decreased trabecular bone area and increased bone mineral density in tibial metaphysis, in rats following short-term TCDD-exposure (Lind et al. 2009b). Further, they showed that the chemical composition of bone was affected, resulting in bone minerals with characteristics of more mature bone, while the degree of mineralization was unaffected (Lind et al. 2009b). The altered bone material and macro-mechanical properties in **paper III**, resulting in harder and stiffer bone matrix, also resemble characteristics of more mature bone. No effect was seen on plasticity index, indicating that the collagen properties, which are contributing the most to the plastic response of the bone (Burstein et al. 1975), were not affected. The expression of genes related to osteogenesis was affected by TCDD-exposure, with mostly down-regulated genes, whereas a few genes were up-regulated (paper III, Figure 3), further suggesting that the observed bone properties following TCDD-exposure might be a result of impaired remodeling.

In comparison with the bone tissue effects following perinatal TCDD-exposure in **paper II**, which resulted in delayed matrix maturation and decreased stiffness, the results in **paper III** suggest that exposure to TCDD in adulthood causes a harder, stiffer and more brittle bone tissue. This is likely to reflect the differences between affected bone modeling and matrix maturation from start, versus impaired remodeling of existing mineralized bone matrix.

**TCDD inhibits late phases of osteoblast differentiation *in vitro***

In **paper V**, TCDD was observed to affect the phases of matrix maturation and mineralization in osteoblastic cells (Figure 13), while no effects on proliferation were observed, which is consistent with previous findings (Korkalainen et al. 2009).

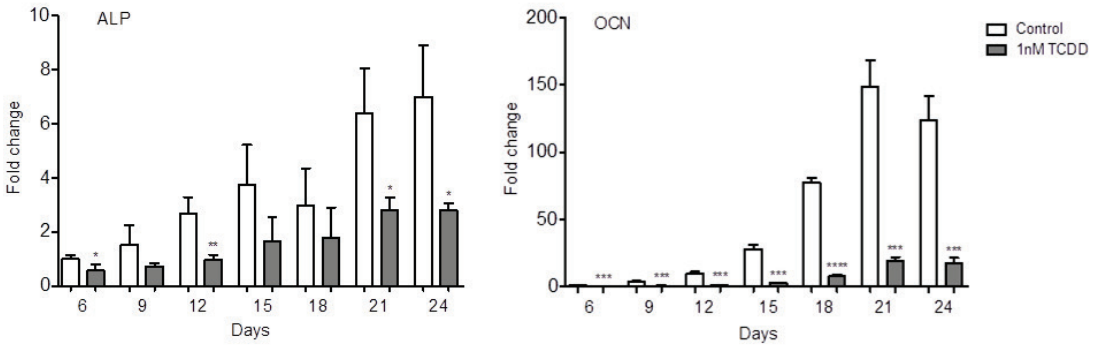


**Figure 13.** Time-course effects of TCDD on the expression of ALP and OCN (paper V).

When comparing exposure only during the first part of differentiation (day 0-6) or only during the later part of differentiation (day 11-20), no differences in effects on the expression of differentiation markers were seen. This finding suggests that the timing of exposure during osteoblast differentiation is not important, although the long half-life of dioxin might contribute to this finding.

It has been suggested that TCDD affects osteoblast differentiation and function by altering the cell architecture, adhesive properties and calcium homeostasis (Carpi et al. 2009). As osteocalcin is implicated in bone mineralization, the finding of decreased osteocalcin expression might suggest consequences for the mineralization process. This is in line with the findings in both **paper II** and **paper III**, which indicates that exposure to TCDD affects the mineralization of bone, although with different outcome depending of the timing of exposure. Perinatal exposure resulted in softer bone, while adult exposure caused harder and stiffer bone. Osteoblasts originate from the mesenchymal stem cells that also give rise to adipocytes and chondrocytes, and it has been demonstrated that these cell lineages have the ability to transdifferentiate, even when already pre-commited, so that cells of the osteoblastic linage could switch to become adipocytes or chondrocytes, and vice versa (Song and Tuan 2004). An especially close inter-relationship has been shown between the osteoblastogenensis and adipogenesis pathways (Beresford et al. 1992; Nuttall and Gimble 2004; Park et al. 1999; Schilling et al. 2007), and these interactions have gained interest in the pathogenesis of osteoporosis (Meunier et al. 1971; Nuttall and Gimble 2004). It could therefore be speculated that exposure to TCDD has the ability to drive the cells towards an adipocyte or chondrocyte lineage pathway in the expense of osteoblasts, thereby affecting mineralization. Another process that may potentially be affected is the transition of osteoblasts into osteocytes. A proportion of osteoblasts at the end of the bone formation phase transforms into osteocytes, which are thought to have important roles in bone remodeling. An inappropriate differentiation of osteocytes could be expected to impact the mineralization of bone, and it has been suggested that accelerated osteoblast-to-osteocyte transition leads to a bone matrix with reduced osteoid and increased mineralization (Laue et al. 2011).

**Aroclor 1254 exhibits dioxin-like bone effects *in-vivo* and *in-vitro***

In the environment, dioxins and dioxin-like compounds are present as mixtures together also with non-dioxin like compounds, and in **paper IV**, bone effects of exposure to the PCB-mixture Arocor 1254, which contains both dioxin-like and non-dioxin-like congeners, were studied. Perinatal exposure to Arocor 1254 affected bone geometry, mineral density and macro-mechanical properties of young rat offspring, while in older offspring, along with discontinued exposure of Aroclor 1254, these effects where no longer detectable. The bone effect pattern elicited by perinatal exposure to Aroclor 1254 was similar to the effects observed by perinatal TCDD-exposure in **paper II**, including decreased cross-sectional area, cortical thickness and cortical bone mineral density, as well as bending force and stiffness. It has been shown that there is not always a sharp border between the effect patterns of dioxin-like and non-dioxin-like PCB-congeners (Elabbas et al. 2013). Based on estimated TCDD equvivalents for the bone effects by Aroclor 1254 compared with the toxic equivalent (TEQ) value calculated from the congener content in the mixture, the results indicate that the effects of Aroclor 1254 were mainly driven by the dioxin-like congeners.

In **paper V**, it was shown that Aroclor 1254 also affects osteoblast differentiation *in vitro* similar to TCDD, as evaluated by the expression of the marker genes RUNX2, ALP and OCN, although the maximal effects are lower and the doses required were higher compared to TCDD. In order to estimate whether only the dioxin-like congeners, or also non-dioxin-like congeners, are driving these effects, relative potency (REP) values calculated from $EC_{50}$-values, were compared to REP-values based on the chemical composition of Aroclor 1254. The REP-values for the decreased expression of ALP and OCN were shown to be similar to, or slightly lower, than the REP-value based on the chemical composition, suggesting, consistent with the *in vivo* results in **paper IV**, that the observed effects are mainly driven by the dioxin-like congeners in Aroclor 1254. This is also illustrated by similarities of the dose-response curves when the doses of TCDD and Aroclor 1254 are expressed as TEQ, visualizing the impact of the dioxin-like components in Aroclor 1254 alone (Figure 14).

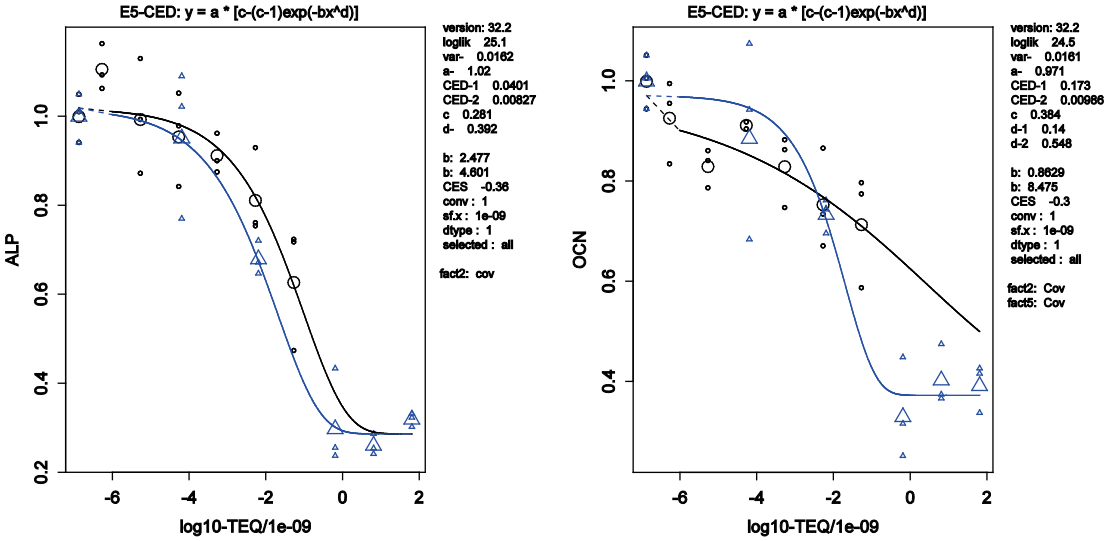


**Figure 14.** Dose-response curves for the decreased expression of A) ALP and B) OCN following Aroclor 1254 (triangles) and TCDD (circles) exposure, with the doses expressed as TEQ (paper VI).

It cannot, however, be excluded that also non-dioxin-like congeners, which act via non-AhR-dependent modes-of-action and therefore are not taken into account in the TEF system, contribute to the exposure outcome. As the estimated potency of Aroclor 1254 was lower than the REP-values based on the dioxin-like constituents in the mixture, such contribution seems to be inhibitory rather than additive or synergistic. In order to elucidate possible contribution of non-dioxin-like components in the mixture, these congeners should be tested also separately. The consistency of the quantitative estimations between the *in vivo* and *in vitro* findings in **papers IV** and **V**, suggests that ALP and OCN expression are useful as markers for dioxin-like effects on bone.

**Dioxin-induced effects on bone differ partly between the genders**

In **paper III**, the effects of TCDD on bone were shown to partly differ between the genders. Effects on the cortical bone structure, such as cortical porosity, as well as parameters describing the material properties, were more pronounced in females, and

also the macro-mechanical properties of the whole bone were mainly seen in females. The effects on trabecular bone micro-structure, and the decrease in trabecular bone area, were seen for both genders, while effects on trabecular bone material properties were more pronounced in males. Not many bone toxicity studies have included investigations of both males and females, and there is no study which did a detailed quantitative evaluation of the data to clarify any possible gender differences due to TCDD-induced bone toxicity. Semi-quantitative and qualitative data analyses resulted in reports on no differences between the genders for bone effects of TCDD following perinatal and lactational exposure (Miettinen et al. 2005), while perinatal and lactational TCDD-exposure of rhesus monkeys resulted in altered bone geometry and composition in females, but only a few macro-mechanical parameters affected in males (Hermsen et al. 2008). Female CA-AhR mice, which have a constitutively active AhR, were shown to differ considerably more in bone phenotype compared to wild-type mice, than did the males (Wejheden et al. 2010). For the bone effects elicited by perinatal exposure to Aroclor 1254 in **paper IV**, there were no gender differences observed, while the dioxin-like PCB126 has been shown to cause somewhat different effects depending on estrogen status (Lind et al. 2004). It is likely that differences in effects of dioxin-like compounds between the genders are due both to hormonal status and to exposure regimens such as timing of exposure. Therefore more detailed evaluation of available data and/or systematic studies are needed to fully elucidate any gender differences in dioxin-induced bone toxicity.

## 4.2 THE ROLE OF AHR

**Dioxin-induced modulations of bone are dependent on the AhR**

Previous studies have indicated that bone toxicity belong to the category of dioxin effects that are dependent on the AhR (Jamsa et al. 2001; Miettinen et al. 2005). The differences between the Long-Evans (L-E) and Han/Wistar (H/W) rat strains in sensitivity to a number of dioxin-induced toxic effects have been ascribed to the structurally aberrant AhR, with an insertion/deletion type of alteration at the 3' end of the coding region of cDNA and a smaller receptor size (Pohjanvirta et al. 1998), exhibited by the more TCDD-resistant H/W strain (Tuomisto et al. 1999). In **paper I**, the sensitivity differences to insults of TCDD on adult bone between the L-E and H/W rat strains were investigated using the BMD methodology. Of the bone parameters where both strains showed effects, there was a 10-fold strain difference for decreased energy absorption of proximal tibia and tibial length, and a 50-fold strain difference for decreased cross-sectional area of proximal tibia, while for six parameters there was no difference between the strains. For many of the analyzed bone parameters, covering both geometry, mineral density and macro-mechanical properties, only the L-E rats showed significant dose-response relationships, while the H/W rats were largely unaffected. In **paper III**, where bone effects of TCDD-exposure were compared for $AhR^{+/+}$ and $AhR^{-/-}$ mice, TCDD caused only a few alterations of the bones of $AhR^{-/-}$ mice. In male $AhR^{-/-}$ mice, the cortical porosity was decreased following TCDD-exposure, which is opposite to the increased cortical porosity seen for TCDD-exposed $AhR^{+/+}$ mice. Neither the material properties nor trabecular micro-structure were affected by TCDD in the $AhR^{-/-}$ mice, and consistently, TCDD did not affect the levels of the bone remodeling markers PINP or CTX. The expression of a few osteogenesis-

related genes was altered by TCDD exposure in $AhR^{-/-}$ mice, but the pattern of affected genes was not the same as for $AhR^{+/+}$ mice. In **paper V**, the marker genes for osteoblast differentiation *in vitro* were decreased at the same doses where the expression of AhR-dependent genes was induced, indicating that these effects occur only when the AhR is activated (paper V, figures 3 and 5). Taken together, these findings strongly support that altered bone tissue properties following dioxin exposure are highly dependent on the AhR.

**Absence of a functional AhR modifies the bone phenotype**

In **paper III**, the impact of the AhR on normal bone phenotype was investigated, and it was found that lack of AhR resulted in slightly modified bone tissue properties, which also partly differed between the genders. Principally, the $AhR^{-/-}$ mice had higher trabecular bone volume fraction with lower trabecular separation and increased number of trabeculae, compared to $AhR^{+/+}$ mice. These properties are similar to the alterations seen following TCDD-exposure of $AhR^{+/+}$ mice, although less pronounced in the $AhR^{-/-}$ mice. For female $AhR^{-/-}$ mice the trabecular bone matrix tended to be softer and more elastic, which is opposite to the TCDD-exposure that caused harder and stiffer bone matrix. Together with the serum levels of bone resorption- and formation markers, these findings are indicative of a higher bone remodeling rate, resulting in undermineralization that makes the bone more elastic and less stiff (Seeman 2003).

## 4.3 THE RETINOID SYSTEM IN DIOXIN-INDUCED BONE TISSUE MODULATIONS

In **paper VI**, the effects on serum levels of retinoids were analysed in the same mice, which were characterized for bone tissue modulations in **paper III**. It was shown that exposure to TCDD increased the serum retinol levels. The TCDD-induced modulations of bone tissue in **paper III** showed several similarities with bone properties that have been reported following retinoid treatment, such as increased trabecular bone mineral density, decreased trabecular bone area, harder and stiffer trabecular bone matrix, as well as reduced cortical thickness and cortical bone mineral density (Hough et al. 1988; Johansson et al. 2002; Kneissel et al. 2005; Li et al. 1989; Lind et al. 2011). However, the bone diameter was not reduced, as often reported following retinoid excess (Hough et al. 1988; Johansson et al. 2002; Lind et al. 2006; Lind et al. 2011). In contrast, exposure of adult rats to TCDD in **paper I** caused reduced cross-sectional area, and also the perinatal exposure to TCDD in **paper II** and to Aroclor 1254 in **paper IV** resulted in smaller cross-sectional area, which is consistent with retinoid excess. These effects might be a result of increased periosteal resorption, but following perinatal exposure it may also reflect a decreased periosteal bone formation during development, leading to thinner bone.

The increased mineral density of the trabecular bone, and the decreased cortical bone mineral density, that were associated with a higher serum retinol level following TCDD-exposure in **paper III**, are in line with findings showing compartment-specific impact of retinoids on bone. Retinoid excess has been suggested to cause increased periosteal bone resorption, but suppressed endosteal bone resorption (Kneissel et al. 2005), as well as inhibition of cortical bone formation and increased endosteal mineralization (Lind et al. 2012). Because of the harder and stiffer bone matrix

following TCDD-exposure in **paper III**, which resembles characteristics of more mature bone, the TCDD-induced increase of trabecular bone might reflect impaired bone remodeling rather than increased endosteal mineralization. This is also in line with the decreased expression of differentiation marker genes in the osteoblastic cells following TCDD-exposure *in vitro*.

The bone phenotype following TCDD-exposure did not fully reproduce the bone properties characteristic for retinoid excess, suggesting that other factors beyond the serum retinol levels are likely to be involved in mediating the observed bone effects. It might be that TCDD-induced alteration of retinoid metabolism and signaling within bone cells, as indicated by the altered expression of retinoid-related genes observed in osteoblastic cells *in vitro*, contribute to the effect pattern, although it is not clear if this is a relevant mechanism also *in vivo*. The increased expression of RALDH3 and decreased expression of CYP26a1 could indicate increased levels of retinoic acid in the cells. However, as exposure to TCDD also resulted in reduced expression of ALP and OCN, while OCN has been reported to be upregulated by retinoic acid (Oliva et al. 1993; Thaweboon et al. 2005), the decreased expression of OCN in the present study might reflect suppression of retinoic acid-induced transcriptional activity (Weston et al. 1995) or decreased retinoic acid-responsiveness (Rushing and Denison 2002) by TCDD, and the alterations in expression of retinoid-related genes being a compensatory mechanism.

A number of studies have reported increased osteoblast differentiation and mineralization by retinoic acid *in vitro* (Malladi et al. 2006; Skillington et al. 2002; Song et al. 2005; Wan et al. 2007; Yamashita et al. 2005), but there are also studies reporting decreased mineralization (Cohen-Tanugi and Forest 1998; Iba et al. 2001; Nuka et al. 1997), as well as different effects depending on the stage of osteoblast differentiation (Nagasawa et al. 2005; Nakayama et al. 1990; Nuka et al. 1997). As retinoic acid is suggested to be involved in regulation of osteoblast and adipocyte differentiation from mesenchymal stem cells (Ding et al. 2001; Hisada et al. 2013; Skillington et al. 2002), it is possible that exposure to potent AhR-ligands, via alterations of the retinoid signaling system, may shift the fate of the mesenchymal stem cells and thereby impact osteogenesis. Further, elevated retinoic acid levels have been reported to promote osteoblast-to-osteocyte transitioning (Laue et al. 2011), and it could be speculated that altered retinoic acid signaling within osteoblastic cells result in an inappropriate osteocyte differentiation, which are expected to impact the bone properties. Furthermore, both stimulatory and inhibitory effects of retinoic acid on different phases of osteoclast differentiation and activity has been shown (Balkan et al. 2011; Conaway et al. 2011; Hu et al. 2010; Kindmark et al. 1995; Saneshige et al. 1995; Scheven and Hamilton 1990; Togari et al. 1991). Thus, also modulations of osteoclasts by retinoid signaling may be influencing the bone remodeling following dioxin exposure.

In addition to AhR-ligands, other environmental chemicals, suspected to belong to the EDC-type of compounds, have the ability to both interact with the retinoid system and to affect bone. The brominated flame retardant hexabromocyclododecane (HBCD) decreased hepatic retinoid levels following perinatal exposure in rats, which also showed decreased trabecular bone mineral density, bone diameter and cortical

thickness (van der Ven et al. 2009). The organotin compound tributyltin (TBT), which is a ligand of the RXR (le Maire et al. 2009), has been shown to delay ossification of the skeleton in rat foetuses following prenatal exposure (Adeeko et al. 2003), and to affect osteoblast (Koskela et al. 2012) and osteoclast (Koskela et al. 2012; Yonezawa et al. 2007) differentiation *in vitro*. Effects on ostoclastogenesis were demonstrated to be inhibited by a RAR antagonist, suggesting involvement of a retinoid dependent pathway for the effects (Yonezawa et al. 2007). These findings, together with the observations in the present study, strengthen the relevance of the retinoid system as an important pathway in bone toxicity by EDCs.

## 4.4 BENCHMARKDOSE MODELING OF BONE PARAMETERS

In **paper I**, the BMD approach was applied for the bone parameters affected by TCDD in order to derive BMDs and to evaluate the usefulness of the approach for quantitative assessment of bone toxicity parameters. The BMDLs corresponding to an effect level of 5% were in general consistent with the corresponding NOAELs, while the 10% effect level resulted in BMDLs higher than the NOAEL values for most parameters. As bone has not traditionally been used as an endpoint in environmental toxicology studies, information about effect levels in relation to adversity of the outcome for the various parameters are scarce. The consistency of the BMDLs with the NOAELs in **paper I** suggests that the BMD approach is appropriate for evaluation of these parameters, and that the 5% effect level is relevant. However, although NOAEL values have been widely used in risk assessment, these values are rough measures of no-effect-levels and highly dependent on the experimental dose levels. The fact that the NOAELs in this study were in the same range as the BMDLs at a 5% effect level, indicates that they did not represent no-effect-levels but rather correspond to an effect size of 5%.

As an approach to evaluate the potency of Aroclor 1254 to induced bone effects in **paper IV**, for which only one dose level was available, the effect size resulting from the actual exposure was used as the CES for deriving BMDs for the same parameters affected by TCDD in a similarly designed study. The resulting ratio between the TCDD equivalents and the theoretical REP values, were consistent with the corresponding results from effects of Aroclor 1254 on osteoblasts *in vitro* in **paper V**, suggesting this approach to be appropriate. Further, in **paper V**, BMDs with a CES of 5% ($BMD_5$) were calculated. For TCDD exposure, the $BMD_5$ values were similar to the doses inducing significant effects as analyzed by ANOVA, while the $BMD_5$ values for Aroclor 1254 were several potencies lower. The $BMD_5$ reflects the point-of-departure of a dose-response curve, and due to the smaller effect size and less steep dose-response curve for Aroclor 1254, the $BMD_5$ method was in this case demonstrated to be more sensitive than ANOVA in identifying the dose-range where the effects start. This is supporting the use of BMD methodology to derive point-of-departure from dose-response curves in bone toxicity studies for use in risk assessment.

# 5 CONCLUSIONS

The quality and strength of bone tissue depends on both its structural and material properties. As any modification of these properties influences bone tissue quality, it is of value to examine the bone at various levels of its different compartments in order to provide insight into the consequences that exposure to various chemicals might have on bone. In this thesis, both geometrical, micro-structural, matrix material and macro-mechanical parameters of the bone have been examined. In addition, cell studies and gene expression analyzes were performed in order to elucidate effects on the osteoblast differentiation process.

From these studies it is clear that bone tissue is affected by exposure to potent AhR-ligands such as dioxins, and that the AhR plays a crucial role for the manifestation of the effects. The presence or absence of a functional AhR also impacts the normal bone phenotype. Further, the outcome of the exposure is clearly influenced by the timing of exposure. Perinatal exposure to TCDD resulted in delayed matrix maturation, while exposure during adulthood caused a harder and stiffer bone matrix. The effect pattern and effect size was also observed to differ partly between the genders.

On the cellular level, osteoblast differentiation was shown to be a target for TCDD-exposure, which is consistent with the observed disturbances of bone mineralization following *in vivo* exposure. Further, exposure of osteoblastic cells to TCDD altered the expression of retinoid-related genes, which might reflect a contributing mode-of-action for the observed bone effect pattern. In addition, effects on bone properties following exposure to TCDD *in vivo* were associated with altered serum retinoid levels, which may influence the bone tissue modulations.

Exposure to the PCB-mixture Aroclor 1254, which contains both dioxin-like and non-dioxin-like congeners, caused a similar pattern of bone alterations as TCDD-exposure alone, both on bone properties *in vivo* and on osteoblastic cells *in vitro*, with the effects being mainly driven by the dioxin-like congeners in the mixture.

Based on the observations in the experimental models in this study, the overall results show that environmental contaminants, to which humans are continuously exposed, have the ability to modulate the osteogenesis process, and suggest that the alterations of bone tissue are relevant endpoints in studies of effects of dioxin exposure. Taken together with data from studies showing bone effects by compounds with different modes-of-action, bone is likely to be useful as a model for studying health effects of chemicals also beyond dioxins.

# 6 FUTURE PERSPECTIVES

This thesis has characterized dioxin-induced bone tissue modulations. Further evaluation of bone toxicity profiles for certain types of mode-of-action is needed in order to establish bone alterations as reliable effect markers for various types of chemicals, and for development of test systems for chemicals with bone-toxic properties.

As the exposure to environmental contaminants is always as combinations of multiple chemicals, whose interactions and impact on each other's toxic effects are mostly unknown, disturbances of bone properties should be further evaluated following combined exposure. For example, interactions between organochlorines and other groups of compounds, such as perfluorinated compounds, as well as metals, are of high relevance for the current exposure situation.

In order to clarify the impact of an altered retinoid signaling for dioxin-induced bone modulations, the expression of retinoid-related genes in bone following dioxin exposure should also be examined *in vivo*. In addition, interactions of the AhR- and retinoid systems with other signaling pathways should be taken into account.

The contribution of a disturbed osteoclastogenesis for the observed effects, and possible effects on the coupling between osteoblast and osteoclast activities, would be of interest to explore.

Based on the inter-relationship between cells of mesenchymal linages, future studies should address the possibility that disturbances of the relation between osteogenesis, adipogenesis and chondrogenesis, are involved in the observed bone effects by environmental chemicals. Also possible effects on osteocyte differentiation should be addressed. Further, increasing evidence links exposure to environmental chemicals to epigenetic alterations, and as epigenetic regulation are shown to contribute to linage-specific differentiation of mesenchymal stem cells, it would be of interest to investigate whether epigenetic modifications of genes regulating osteogenesis could be an underlying mechanism for the affected bone tissue properties.

# 7 ACKNOWLEDGEMENTS

This thesis has been performed at the Institute of Environmental Medicine, Karolinska Institutet. I would like to thank all of those who, directly or indirectly, contributed to this work. In particular I would like to thank:

Helen for taking me on as a PhD student, for the opportunity to participate actively in EU projects and to be involved in various other projects – this has really given valuable experiences. Thanks for your patience and support, and your interest in developing this thesis project into new areas.

Matti for always having valuable input and advises, for letting me visit your lab to learn the bone cell work, and for your hospitality during my stay in Kuopio.

Rachel for inspiration and support, and for advises on the cell exposure studies.

Bertrand for generously providing lab space and facilities at CCK, and for valuable input.

Merja for introducing me to the bone cell work, and for being available to answer questions throughout this project. Thanks also for your hospitality during my stay in Kuopio.

Kina for all the help in the lab, and for always being there for a talk.

Sabina for being a great room mate and friend, for "fika" and chocolate, and for always inspiring conversations.

Hanna for introducing me in the lab during my master thesis project, and Natasha for introducing the pQCT-technique. Mikko for your interest in applying new methods for analyses of bone tissue, and for demonstrating the nanoindentation technique in Shrivenham. Juha for a pleasant stay in your lab for the bone biomechanics work, and for guidance of skiing tracks in Oulo. Joakim and Mattias for your contribution to paper V. Javier for contribution to paper VI. Ulrika, Pinelopi, Edel, Naveen, Johanna, Jens and Miguel for generously sharing knowledge and equipment in your lab. Lotta and Annette for nice lunches and talks.

All present and past members of the Environmental Health Risk Assessment Unit. Thanks to Robert, Lubna, Daniel, Anna, Ali, Jianyao, Lotta, Annika, Johanna, Per, Emma, Lina, and all the rest for providing a nice atmosphere.

Everyone at floor 3, and the rest of IMM, who have contributed in one way or another.

And my family:

Dennis for having been such a happy, joyful friend. Ann & Leif för att ni tagit så väl hand om Dennis under mina studier. Terese & Andreas for your company, and for being always supporting.

Reno for your encouragement and support, for all the good coffee, and for sharing your life with me!

# 8 REFERENCES


Adeeko, A., Li, D., Forsyth, D.S., Casey, V., Cooke, G.M., Barthelemy, J., Cyr, D.G., Trasler, J.M., Robaire, B. and Hales, B.F. 2003. Effects of in utero tributyltin chloride exposure in the rat on pregnancy outcome. Toxicol Sci 74, 407-415.

Alvarez-Lloret, P., Lind, P.M., Nyberg, I., Orberg, J. and Rodriguez-Navarro, A.B. 2009. Effects of 3,3',4,4',5-pentachlorobiphenyl (PCB126) on vertebral bone mineralization and on thyroxin and vitamin D levels in Sprague-Dawley rats. Toxicol Lett 187, 63-68.

Alveblom, A.K., Rylander, L., Johnell, O. and Hagmar, L. 2003. Incidence of hospitalized osteoporotic fractures in cohorts with high dietary intake of persistent organochlorine compounds. Int Arch Occup Environ Health 76, 246-248.

An, J., Zou, W., Zhong, Y., Zhang, X., Wu, M., Yu, Z. and Ye, T. 2012. The toxic effects of Aroclor 1254 exposure on the osteoblastic cell line MC3T3-E1 and its molecular mechanism. Toxicology 295, 8-14.

Andreola, F., Fernandez-Salguero, P.M., Chiantore, M.V., Petkovich, M.P., Gonzalez, F.J. and De Luca, L.M. 1997. Aryl hydrocarbon receptor knockout mice (AHR-/-) exhibit liver retinoid accumulation and reduced retinoic acid metabolism. Cancer Res 57, 2835-2838.

Andrews, J.E. 1989. Polychlorinated biphenyl (Aroclor 1254) induced changes in femur morphometry calcium metabolism and nephrotoxicity. Toxicology 57, 83-96.

Balkan, W., Rodriguez-Gonzalez, M., Pang, M., Fernandez, I. and Troen, B.R. 2011. Retinoic acid inhibits NFATc1 expression and osteoclast differentiation. J Bone Miner Metab 29, 652-661.

Balmer, J.E. and Blomhoff, R. 2002. Gene expression regulation by retinoic acid. J Lipid Res 43, 1773-1808.

Beard, J., Marshall, S., Jong, K., Newton, R., Triplett-McBride, T., Humphries, B. and Bronks, R. 2000. 1,1,1-trichloro-2,2-bis (p-chlorophenyl)-ethane (DDT) and reduced bone mineral density. Arch Environ Health 55, 177-180.

Bengtsson, B.E., Larsson, A., Bengtsson, A. and Renberg, L. 1988. Sublethal effects of tetrachloro-1,2-benzoquinone--a component in bleachery effluents from pulp mills--on vertebral quality and physiological parameters in fourhorn sculpin. Ecotoxicol Environ Saf 15, 62-71.

Beresford, J.N., Bennett, J.H., Devlin, C., Leboy, P.S. and Owen, M.E. 1992. Evidence for an inverse relationship between the differentiation of adipocytic and osteogenic cells in rat marrow stromal cell cultures. J Cell Sci 102 ( Pt 2), 341-351.

Bergman, A., Olsson, M. and Reiland, S. 1992. Skull-Bone Lesions in the Baltic Gray Seal (Halichoerus-Grypus). Ambio 21, 517-519.

Blomhoff, R. and Blomhoff, H.K. 2006. Overview of retinoid metabolism and function. J Neurobiol 66, 606-630.

Bock, K.W. and Kohle, C. 2006. Ah receptor: dioxin-mediated toxic responses as hints to deregulated physiologic functions. Biochem Pharmacol 72, 393-404.

Bohannon, A.D., Cooper, G.S., Wolff, M.S. and Meier, D.E. 2000. Exposure to 1,1-dichloro-2,2-bis(p-chlorophenyl)ethylene (DDT) in relation to bone mineral density and rate of bone loss in menopausal women. Arch Environ Health 55, 386-391.

Brunnberg, S., Andersson, P., Lindstam, M., Paulson, I., Poellinger, L. and Hanberg, A. 2006. The constitutively active Ah receptor (CA-Ahr) mouse as a potential model for dioxin exposure--effects in vital organs. Toxicology 224, 191-201.

Burstein, A.H., Zika, J.M., Heiple, K.G. and Klein, L. 1975. Contribution of collagen and mineral to the elastic-plastic properties of bone. J Bone Joint Surg Am 57, 956-961.

Carpi, D., Korkalainen, M., Airoldi, L., Fanelli, R., Hakansson, H., Muhonen, V., Tuukkanen, J., Viluksela, M. and Pastorelli, R. 2009. Dioxin-sensitive proteins in differentiating osteoblasts: effects on bone formation in vitro. Toxicol Sci 108, 330-343.

Cohen-Tanugi, A. and Forest, N. 1998. Retinoic acid suppresses the osteogenic differentiation capacity of murine osteoblast-like 3/A/1D-1M cell cultures. Differentiation 63, 115-123.

Conaway, H.H. and Lerner, U.H. 2011. Retinoids and bone. In: E. Diamanti-Kandarakis (Ed), Contemporary aspects of endocrinology, In Tech, pp. 443-454.

Conaway, H.H., Pirhayati, A., Persson, E., Pettersson, U., Svensson, O., Lindholm, C., Henning, P., Tuckermann, J. and Lerner, U.H. 2011. Retinoids stimulate periosteal bone resorption by enhancing the protein RANKL, a response inhibited by monomeric glucocorticoid receptor. J Biol Chem 286, 31425-31436.

Cote, S., Ayotte, P., Dodin, S., Blanchet, C., Mulvad, G., Petersen, H.S., Gingras, S. and Dewailly, E. 2006. Plasma organochlorine concentrations and bone ultrasound measurements: a cross-sectional study in peri-and postmenopausal Inuit women from Greenland. Environ Health 5, 33.

Cullinane, D.M. 2002. The role of osteocytes in bone regulation: mineral homeostasis versus mechanoreception. J Musculoskelet Neuronal Interact 2, 242-244.

Davarinos, N.A. and Pollenz, R.S. 1999. Aryl hydrocarbon receptor imported into the nucleus following ligand binding is rapidly degraded via the cytosplasmic proteasome following nuclear export. J Biol Chem 274, 28708-28715.

Denison, M.S. and Nagy, S.R. 2003. Activation of the aryl hydrocarbon receptor by structurally diverse exogenous and endogenous chemicals. Annu Rev Pharmacol Toxicol 43, 309-334.

Dhem, A. and Goret-Nicaise, M. 1984. Effects of retinoic acid on rat bone. Food Chem Toxicol 22, 199-206.

Ding, J., Woo, J.T. and Nagai, K. 2001. The effects of retinoic acid on reversing the adipocyte differentiation into an osteoblastic tendency in ST2 cells, a murine bone marrow-derived stromal cell line. Cytotechnology 36, 125-136.

Dong, W., Hinton, D.E. and Kullman, S.W. 2012. TCDD disrupts hypural skeletogenesis during medaka embryonic development. Toxicol Sci 125, 91-104.

Ducy, P. 2000. Cbfa1: a molecular switch in osteoblast biology. Dev Dyn 219, 461-471.

EFSA. 2009. Use of the benchmark dose approach in risk assessment. Guidance of the Scientific Committee. The EFSA Journal 1150, 1-72.

Elabbas, L.E., Westerholm, E., Roos, R., Halldin, K., Korkalainen, M., Viluksela, M. and Håkansson, H. 2013. Non Dioxin-Like Polychlorinated Biphenyl: exposure and health hazards. In: M. Rose and A. Fernandes (Eds), Persistent Organic Pollutants and Toxic Metals in Foods, Woodhead publishing Ltd, pp. 215-260.

Feretti, J.L. 1999. Peripheral quantitative computed tomography (pQCT) for evaluating structural and mechanical properties of small bones. In: Y.H. An and R.A. Draughn (Eds), Practical guide for mechanical testing of bones, CRC Press, pp. 2-25.

Fernandez-Salguero, P., Pineau, T., Hilbert, D.M., McPhail, T., Lee, S.S., Kimura, S., Nebert, D.W., Rudikoff, S., Ward, J.M. and Gonzalez, F.J. 1995. Immune system impairment and hepatic fibrosis in mice lacking the dioxin-binding Ah receptor. Science 268, 722-726.

Feskanich, D., Singh, V., Willett, W.C. and Colditz, G.A. 2002. Vitamin A intake and hip fractures among postmenopausal women. JAMA 287, 47-54.

Finnila, M.A., Zioupos, P., Herlin, M., Miettinen, H.M., Simanainen, U., Hakansson, H., Tuukkanen, J., Viluksela, M. and Jamsa, T. 2010. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin exposure on bone material properties. J Biomech 43, 1097-1103.

Fletcher, N., Giese, N., Schmidt, C., Stern, N., Lind, P.M., Viluksela, M., Tuomisto, J.T., Tuomisto, J., Nau, H. and Hakansson, H. 2005. Altered retinoid metabolism in female Long-Evans and Han/Wistar rats following long-term 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD)-treatment. Toxicol Sci 86, 264-272.

Fox, G.A., Lundberg, R., Wejheden, C., Lind, L., Larsson, S., Orberg, J. and Lind, P.M. 2008. Health of herring gulls (Larus argentatus) in relation to breeding location in the early 1990s. III. Effects on the bone tissue. J Toxicol Environ Health A 71, 1448-1456.

Gasser, J.A. 1995. Assessing bone quantity by pQCT. Bone 17, 145S-154S.

Gierthy, J.F., Silkworth, J.B., Tassinari, M., Stein, G.S. and Lian, J.B. 1994. 2,3,7,8-Tetrachlorodibenzo-p-dioxin inhibits differentiation of normal diploid rat osteoblasts in vitro. J Cell Biochem 54, 231-238.

Glynn, A.W., Michaelsson, K., Lind, P.M., Wolk, A., Aune, M., Atuma, S., Darnerud, P.O. and Mallmin, H. 2000. Organochlorines and bone mineral density in Swedish men from the general population. Osteoporos Int 11, 1036-1042.

Gonzalez, F.J. and Fernandez-Salguero, P. 1998. The aryl hydrocarbon receptor: studies using the AHR-null mice. Drug Metab Dispos 26, 1194-1198.

Gutleb, A.C., Arvidsson, D., Orberg, J., Larsson, S., Skaare, J.U., Aleksandersen, M., Ropstad, E. and Lind, P.M. 2010. Effects on bone tissue in ewes (Ovies aries) and their foetuses exposed to PCB 118 and PCB 153. Toxicol Lett 192, 126-133.

Hahn, M.E., Allan, L.L. and Sherr, D.H. 2009. Regulation of constitutive and inducible AHR signaling: complex interactions involving the AHR repressor. Biochem Pharmacol 77, 485-497.

Hankinson, O. 1995. The aryl hydrocarbon receptor complex. Annu Rev Pharmacol Toxicol 35, 307-340.

Heid, S.E., Pollenz, R.S. and Swanson, H.I. 2000. Role of heat shock protein 90 dissociation in mediating agonist-induced activation of the aryl hydrocarbon receptor. Mol Pharmacol 57, 82-92.

Heino, T.J., Kurata, K., Higaki, H. and Vaananen, H.K. 2009. Evidence for the role of osteocytes in the initiation of targeted remodeling. Technol Health Care 17, 49-56.

Herlin, M., Kalantari, F., Stern, N., Sand, S., Larsson, S., Viluksela, M., Tuomisto, J.T., Tuomisto, J., Tuukkanen, J., Jamsa, T., Lind, P.M. and Hakansson, H. 2010. Quantitative characterization of changes in bone geometry, mineral density and biomechanical properties in two rat strains with different Ah-receptor structures after long-term exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Toxicology 273, 1-11.

Hermsen, S.A., Larsson, S., Arima, A., Muneoka, A., Ihara, T., Sumida, H., Fukusato, T., Kubota, S., Yasuda, M. and Lind, P.M. 2008. In utero and lactational exposure to 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD) affects bone tissue in rhesus monkeys. Toxicology 253, 147-152.

Hisada, K., Hata, K., Ichida, F., Matsubara, T., Orimo, H., Nakano, T., Yatani, H., Nishimura, R. and Yoneda, T. 2013. Retinoic acid regulates commitment of undifferentiated mesenchymal stem cells into osteoblasts and adipocytes. J Bone Miner Metab 31, 53-63.

Hodgson, S., Thomas, L., Fattore, E., Lind, P.M., Alfven, T., Hellstrom, L., Hakansson, H., Carubelli, G., Fanelli, R. and Jarup, L. 2008. Bone mineral density changes in relation to environmental PCB exposure. Environ Health Perspect 116, 1162-1166.

Hofbauer, L.C., Khosla, S., Dunstan, C.R., Lacey, D.L., Boyle, W.J. and Riggs, B.L. 2000. The roles of osteoprotegerin and osteoprotegerin ligand in the paracrine regulation of bone resorption. J Bone Miner Res 15, 2-12.

Hoffman, D.J., Melancon, M.J., Klein, P.N., Rice, C.P., Eisemann, J.D., Hines, R.K., Spann, J.W. and Pendleton, G.W. 1996. Developmental toxicity of PCB 126 (3,3',4,4',5-pentachlorobiphenyl) in nestling American kestrels (Falco sparverius). Fundam Appl Toxicol 34, 188-200.

Holliday, D.K. and Holliday, C.M. 2012. The effects of the organopollutant PCB 126 on bone density in juvenile diamondback terrapins (Malaclemys terrapin). Aquat Toxicol 109, 228-233.

Hornung, M.W., Spitsbergen, J.M. and Peterson, R.E. 1999. 2,3,7,8-Tetrachlorodibenzo-p-dioxin alters cardiovascular and craniofacial development and function in sac fry of rainbow trout (Oncorhynchus mykiss). Toxicol Sci 47, 40-51.

Hough, S., Avioli, L.V., Muir, H., Gelderblom, D., Jenkins, G., Kurasi, H., Slatopolsky, E., Bergfeld, M.A. and Teitelbaum, S.L. 1988. Effects of hypervitaminosis A on the bone and mineral metabolism of the rat. Endocrinology 122, 2933-2939.

Hu, L., Lind, T., Sundqvist, A., Jacobson, A. and Melhus, H. 2010. Retinoic acid increases proliferation of human osteoclast progenitors and inhibits RANKL-stimulated osteoclast differentiation by suppressing RANK. PLoS One 5, e13305.

Iba, K., Chiba, H., Yamashita, T., Ishii, S. and Sawada, N. 2001. Phase-independent inhibition by retinoic acid of mineralization correlated with loss of tetranectin expression in a human osteoblastic cell line. Cell Struct Funct 26, 227-233.

Ikuta, T., Tachibana, T., Watanabe, J., Yoshida, M., Yoneda, Y. and Kawajiri, K. 2000. Nucleocytoplasmic shuttling of the aryl hydrocarbon receptor. J Biochem 127, 503-509.

Ilvesaro, J., Pohjanvirta, R., Tuomisto, J., Viluksela, M. and Tuukkanen, J. 2005. Bone resorption by aryl hydrocarbon receptor-expressing osteoclasts is not disturbed by TCDD in short-term cultures. Life Sci 77, 1351-1366.

Jacobs, H., Dennefeld, C., Feret, B., Viluksela, M., Hakansson, H., Mark, M. and Ghyselinck, N.B. 2011. Retinoic acid drives aryl hydrocarbon receptor expression and is instrumental to dioxin-induced toxicity during palate development. Environ Health Perspect 119, 1590-1595.

Jamsa, T., Viluksela, M., Tuomisto, J.T., Tuomisto, J. and Tuukkanen, J. 2001. Effects of 2,3,7,8-tetrachlorodibenzo-p-dioxin on bone in two rat strains with different aryl hydrocarbon receptor structures. J Bone Miner Res 16, 1812-1820.

Jaworski, Z.F. 1981. Physiology and pathology of bone remodeling. Cellular basis of bone structure in health and in osteoporosis. Orthop Clin North Am 12, 485-512.

Johansson, S., Lind, P.M., Hakansson, H., Oxlund, H., Orberg, J. and Melhus, H. 2002. Subclinical hypervitaminosis A causes fragile bones in rats. Bone 31, 685-689.

Kawamura, T. and Yamashita, I. 2002. Aryl hydrocarbon receptor is required for prevention of blood clotting and for the development of vasculature and bone in the embryos of medaka fish, Oryzias latipes. Zoolog Sci 19, 309-319.

Kindmark, A., Melhus, H., Ljunghall, S. and Ljunggren, O. 1995. Inhibitory effects of 9-cis and all-trans retinoic acid on 1,25(OH)2 vitamin D3-induced bone resorption. Calcif Tissue Int 57, 242-244.

Kindmark, A., Torma, H., Johansson, A., Ljunghall, S. and Melhus, H. 1993. Reverse transcription-polymerase chain reaction assay demonstrates that the 9-cis retinoic acid receptor alpha is expressed in human osteoblasts. Biochem Biophys Res Commun 192, 1367-1372.

Kneissel, M., Studer, A., Cortesi, R. and Susa, M. 2005. Retinoid-induced bone thinning is caused by subperiosteal osteoclast activity in adult rodents. Bone 36, 202-214.

Korkalainen, M., Kallio, E., Olkku, A., Nelo, K., Ilvesaro, J., Tuukkanen, J., Mahonen, A. and Viluksela, M. 2009. Dioxins interfere with differentiation of osteoblasts and osteoclasts. Bone 44, 1134-1142.

Koskela, A., Viluksela, M., Keinanen, M., Tuukkanen, J. and Korkalainen, M. 2012. Synergistic effects of tributyltin and 2,3,7,8-tetrachlorodibenzo-p-dioxin on differentiating osteoblasts and osteoclasts. Toxicol Appl Pharmacol 263, 210-217.

Laue, K., Pogoda, H.M., Daniel, P.B., van Haeringen, A., Alanay, Y., von Ameln, S., Rachwalski, M., Morgan, T., Gray, M.J., Breuning, M.H., Sawyer, G.M., Sutherland-Smith, A.J., Nikkels, P.G., Kubisch, C., Bloch, W., Wollnik, B., Hammerschmidt, M. and Robertson, S.P. 2011. Craniosynostosis and multiple skeletal anomalies in humans and zebrafish result from a defect in the localized degradation of retinoic acid. Am J Hum Genet 89, 595-606.

le Maire, A., Grimaldi, M., Roecklin, D., Dagnino, S., Vivat-Hannah, V., Balaguer, P. and Bourguet, W. 2009. Activation of RXR-PPAR heterodimers by organotin environmental endocrine disruptors. EMBO Rep 10, 367-373.

Leppanen, O., Sievanen, H., Jokihaara, J., Pajamaki, I. and Jarvinen, T.L. 2006. Three-point bending of rat femur in the mediolateral direction: introduction and validation of a novel biomechanical testing protocol. J Bone Miner Res 21, 1231-1237.

Li, X.F., Dawson-Hughes, B., Hopkins, R., Russell, R.M., Jee, W.S., Bankson, D. and Li, X.J. 1989. The effects of chronic vitamin A excess on bone remodeling in aged rats. Proc Soc Exp Biol Med 191, 103-107.

Lian, J.B. and Stein, G.S. 1995. Development of the osteoblast phenotype: molecular mechanisms mediating osteoblast growth and differentiation. Iowa Orthop J 15, 118-140.

Lind, P.M., Bergman, A., Olsson, M. and Orberg, J. 2003. Bone mineral density in male Baltic grey seal (Halichoerus grypus). Ambio 32, 385-388.

Lind, P.M., Eriksen, E.F., Lind, L., Orberg, J. and Sahlin, L. 2004. Estrogen supplementation modulates effects of the endocrine disrupting pollutant PCB126 in rat bone and uterus: diverging effects in ovariectomized and intact animals. Toxicology 199, 129-136.

Lind, P.M., Eriksen, E.F., Sahlin, L., Edlund, M. and Orberg, J. 1999. Effects of the antiestrogenic environmental pollutant 3,3',4,4', 5-pentachlorobiphenyl (PCB #126) in rat bone and uterus: diverging effects in ovariectomized and intact animals. Toxicol Appl Pharmacol 154, 236-244.

Lind, P.M., Gustafsson, M., Hermsen, S.A., Larsson, S., Kyle, C.E., Orberg, J. and Rhind, S.M. 2009a. Exposure to pastures fertilised with sewage sludge disrupts bone tissue homeostasis in sheep. Sci Total Environ 407, 2200-2208.

Lind, P.M., Johansson, S., Ronn, M. and Melhus, H. 2006. Subclinical hypervitaminosis A in rat: measurements of bone mineral density (BMD) do not reveal adverse skeletal changes. Chem Biol Interact 159, 73-80.

Lind, P.M., Larsson, S., Oxlund, H., Hakansson, H., Nyberg, K., Eklund, T. and Orberg, J. 2000. Change of bone tissue composition and impaired bone strength in rats exposed to 3,3',4,4',5-pentachlorobiphenyl (PCB126). Toxicology 150, 41-51.

Lind, P.M., Oberg, D., Larsson, S., Kyle, C.E., Orberg, J. and Rhind, S.M. 2010. Pregnant ewes exposed to multiple endocrine disrupting pollutants through sewage sludge-fertilized pasture show an anti-estrogenic effect in their trabecular bone. Sci Total Environ 408, 2340-2346.

Lind, P.M., Wejheden, C., Lundberg, R., Alvarez-Lloret, P., Hermsen, S.A., Rodriguez-Navarro, A.B., Larsson, S. and Rannug, A. 2009b. Short-term exposure to dioxin impairs bone tissue in male rats. Chemosphere 75, 680-684.

Lind, T., Hu, L., Lind, P.M., Sugars, R., Andersson, G., Jacobson, A. and Melhus, H. 2012. Microarray profiling of diaphyseal bone of rats suffering from hypervitaminosis A. Calcif Tissue Int 90, 219-229.

Lind, T., Lind, P.M., Jacobson, A., Hu, L., Sundqvist, A., Risteli, J., Yebra-Rodriguez, A., Larsson, S., Rodriguez-Navarro, A., Andersson, G. and Melhus, H. 2011. High dietary intake of retinol leads to bone marrow hypoxia and diaphyseal endosteal mineralization in rats. Bone 48, 496-506.

Lundberg, R., Jenssen, B.M., Leiva-Presa, A., Ronn, M., Hernhag, C., Wejheden, C., Larsson, S., Orberg, J. and Lind, P.M. 2007. Effects of short-term exposure to the DDT metabolite p,p'-DDE on bone tissue in male common frog (Rana temporaria). J Toxicol Environ Health A 70, 614-619.

Malladi, P., Xu, Y., Yang, G.P. and Longaker, M.T. 2006. Functions of vitamin D, retinoic acid, and dexamethasone in mouse adipose-derived mesenchymal cells. Tissue Eng 12, 2031-2040.

Marks, S.C. and Odgren, P.R. 2002. Structure and development of the skeleton. In: J.P. Bilezikian, L.G. Raisz and G.A. Rodan (Eds), Principles of bone bioogy, Academic press, San Diego, pp. 3-15.

Melhus, H., Michaelsson, K., Kindmark, A., Bergstrom, R., Holmberg, L., Mallmin, H., Wolk, A. and Ljunghall, S. 1998. Excessive dietary intake of vitamin A is associated with reduced bone mineral density and increased risk for hip fracture. Ann Intern Med 129, 770-778.

Meunier, P., Aaron, J., Edouard, C. and Vignon, G. 1971. Osteoporosis and the replacement of cell populations of the marrow by adipose tissue. A quantitative study of 84 iliac bone biopsies. Clin Orthop Relat Res 80, 147-154.

Michaelsson, K., Lithell, H., Vessby, B. and Melhus, H. 2003. Serum retinol levels and the risk of fracture. N Engl J Med 348, 287-294.

Miettinen, H.M., Pulkkinen, P., Jamsa, T., Koistinen, J., Simanainen, U., Tuomisto, J., Tuukkanen, J. and Viluksela, M. 2005. Effects of in utero and lactational TCDD exposure on bone development in differentially sensitive rat lines. Toxicol Sci 85, 1003-1012.

Miller, S.C., de Saint-Georges, L., Bowman, B.M. and Jee, W.S. 1989. Bone lining cells: structure and function. Scanning Microsc 3, 953-960; discussion 960-951.

Mimura, J., Ema, M., Sogawa, K. and Fujii-Kuriyama, Y. 1999. Identification of a novel mechanism of regulation of Ah (dioxin) receptor function. Genes Dev 13, 20-25.

Murphy, K.A., Quadro, L. and White, L.A. 2007. The intersection between the aryl hydrocarbon receptor (AhR)- and retinoic acid-signaling pathways. Vitam Horm 75, 33-67.

Murtomaa, M., Tervaniemi, O.M., Parviainen, J., Ruokojarvi, P., Tuukkanen, J. and Viluksela, M. 2007. Dioxin exposure in contaminated sawmill area: the use of molar teeth and bone of bank vole (Clethrionomys glareolus) and field vole (Microtus agrestis) as biomarkers. Chemosphere 68, 951-957.

Nagasawa, H., Takahashi, S., Kobayashi, A., Tazawa, H., Tashima, Y. and Sato, K. 2005. Effect of retinoic acid on murine preosteoblastic MC3T3-E1 cells. J Nutr Sci Vitaminol (Tokyo) 51, 311-318.

Nakayama, Y., Takahashi, K., Noji, S., Muto, K., Nishijima, K. and Taniguchi, S. 1990. Functional modes of retinoic acid in mouse osteoblastic clone MC3T3-E1, proved as a target cell for retinoic acid. FEBS Lett 261, 93-96.

Naruse, M., Ishihara, Y., Miyagawa-Tomita, S., Koyama, A. and Hagiwara, H. 2002. 3-Methylcholanthrene, which binds to the arylhydrocarbon receptor, inhibits proliferation and differentiation of osteoblasts in vitro and ossification in vivo. Endocrinology 143, 3575-3581.

Naruse, M., Otsuka, E., Ishihara, Y., Miyagawa-Tomita, S. and Hagiwara, H. 2004. Inhibition of osteoclast formation by 3-methylcholanthrene, a ligand for arylhydrocarbon receptor: suppression of osteoclast differentiation factor in osteogenic cells. Biochem Pharmacol 67, 119-127.

Nguyen, L.P. and Bradfield, C.A. 2008. The search for endogenous activators of the aryl hydrocarbon receptor. Chem Res Toxicol 21, 102-116.

Nilsson, C.B. and Hakansson, H. 2002. The retinoid signaling system--a target in dioxin toxicity. Crit Rev Toxicol 32, 211-232.

Nishimura, N., Nishimura, H., Ito, T., Miyata, C., Izumi, K., Fujimaki, H. and Matsumura, F. 2009. Dioxin-induced up-regulation of the active form of vitamin D is the main cause for its inhibitory action on osteoblast activities, leading to developmental bone toxicity. Toxicol Appl Pharmacol 236, 301-309.

Nishimura, N., Yonemoto, J., Miyabara, Y., Fujii-Kuriyama, Y. and Tohyama, C. 2005. Altered thyroxin and retinoid metabolic response to 2,3,7,8-tetrachlorodibenzo-p-dioxin in aryl hydrocarbon receptor-null mice. Arch Toxicol 79, 260-267.

Novak, J., Benisek, M. and Hilscherova, K. 2008. Disruption of retinoid transport, metabolism and signaling by environmental pollutants. Environ Int 34, 898-913.

Nuka, S., Sawada, N., Iba, K., Chiba, H., Ishii, S. and Mori, M. 1997. All-trans retinoic acid inhibits dexamethasone-induced ALP activity and mineralization in human osteoblastic cell line SV HFO. Cell Struct Funct 22, 27-32.

Nuttall, M.E. and Gimble, J.M. 2004. Controlling the balance between osteoblastogenesis and adipogenesis and the consequent therapeutic implications. Curr Opin Pharmacol 4, 290-294.

OECD. 2011. Draft detailed review pater state of teh science on novel in vitro and in vivo screening and testing methods and endpoints for evaluating endocrine disruptors. RTI International.

Oliva, A., Della Ragione, F., Fratta, M., Marrone, G., Palumbo, R. and Zappia, V. 1993. Effect of retinoic acid on osteocalcin gene expression in human osteoblasts. Biochem Biophys Res Commun 191, 908-914.

Opotowsky, A.R. and Bilezikian, J.P. 2004. Serum vitamin A concentration and the risk of hip fracture among women 50 to 74 years old in the United States: a prospective analysis of the NHANES I follow-up study. Am J Med 117, 169-174.

Ozcivici, E., Ferreri, S., Qin, Y.X. and Judex, S. 2008. Determination of bone's mechanical matrix properties by nanoindentation. Methods Mol Biol 455, 323-334.

Park, S.R., Oreffo, R.O. and Triffitt, J.T. 1999. Interconversion potential of cloned human marrow adipocytes in vitro. Bone 24, 549-554.

Petrulis, J.R. and Perdew, G.H. 2002. The role of chaperone proteins in the aryl hydrocarbon receptor core complex. Chem Biol Interact 141, 25-40.

Pohjanvirta, R., Wong, J.M., Li, W., Harper, P.A., Tuomisto, J. and Okey, A.B. 1998. Point mutation in intron sequence causes altered carboxyl-terminal structure in the aryl hydrocarbon receptor of the most 2,3,7,8-tetrachlorodibenzo-p-dioxin-resistant rat strain. Mol Pharmacol 54, 86-93.

Pollenz, R.S. 1996. The aryl-hydrocarbon receptor, but not the aryl-hydrocarbon receptor nuclear translocator protein, is rapidly depleted in hepatic and nonhepatic culture cells exposed to 2,3,7,8-tetrachlorodibenzo-p-dioxin. Molecular Pharmacology 49, 391-398.

Promislow, J.H., Goodman-Gruen, D., Slymen, D.J. and Barrett-Connor, E. 2002. Retinol intake and bone mineral density in the elderly: the Rancho Bernardo Study. J Bone Miner Res 17, 1349-1358.

Puga, A., Ma, C. and Marlowe, J.L. 2009. The aryl hydrocarbon receptor cross-talks with multiple signal transduction pathways. Biochem Pharmacol 77, 713-722.

Quarles, L.D., Yohay, D.A., Lever, L.W., Caton, R. and Wenstrup, R.J. 1992. Distinct proliferative and differentiated stages of murine MC3T3-E1 cells in culture: an in vitro model of osteoblast development. J Bone Miner Res 7, 683-692.

Ramajayam, G., Sridhar, M., Karthikeyan, S., Lavanya, R., Veni, S., Vignesh, R.C., Ilangovan, R., Djody, S.S., Gopalakrishnan, V., Arunakaran, J. and Srinivasan, N. 2007. Effects of Aroclor 1254 on femoral bone metabolism in adult male Wistar rats. Toxicology 241, 99-105.

Reyes, H., Reisz-Porszasz, S. and Hankinson, O. 1992. Identification of the Ah receptor nuclear translocator protein (Arnt) as a component of the DNA binding form of the Ah receptor. Science 256, 1193-1195.

Rho, J.Y., Kuhn-Spearing, L. and Zioupos, P. 1998. Mechanical properties and the hierarchical structure of bone. Med Eng Phys 20, 92-102.

Rodriguez-Navarro, A.B., Romanek, C.S., Alvarez-Lloret, P. and Gaines, K.F. 2006. Effect of in ovo exposure to PCBs and Hg on Clapper Rail bone mineral chemistry from a contaminated salt marsh in coastal Georgia. Environ Sci Technol 40, 4936-4942.

Roos, A., Riget, F. and Orberg, J. 2010. Bone mineral density in Swedish otters (Lutra lutra) in relation to PCB and DDE concentrations. Ecotoxicol Environ Saf 73, 1063-1070.

Rushing, S.R. and Denison, M.S. 2002. The silencing mediator of retinoic acid and thyroid hormone receptors can interact with the aryl hydrocarbon (Ah) receptor but fails to repress Ah receptor-dependent gene expression. Arch Biochem Biophys 403, 189-201.

Ryan, E.P., Holz, J.D., Mulcahey, M., Sheu, T.J., Gasiewicz, T.A. and Puzas, J.E. 2007. Environmental toxicants may modulate osteoblast differentiation by a mechanism involving the aryl hydrocarbon receptor. J Bone Miner Res 22, 1571-1580.

Sand, S., Victorin, K. and Filipsson, A.F. 2008. The current state of knowledge on the use of the benchmark dose concept in risk assessment. J Appl Toxicol 28, 405-421.

Saneshige, S., Mano, H., Tezuka, K., Kakudo, S., Mori, Y., Honda, Y., Itabashi, A., Yamada, T., Miyata, K., Hakeda, Y. and et al. 1995. Retinoic acid directly stimulates osteoclastic bone resorption and gene expression of cathepsin K/OC-2. Biochem J 309 ( Pt 3), 721-724.

Scheven, B.A. and Hamilton, N.J. 1990. Retinoic acid and 1,25-dihydroxyvitamin D3 stimulate osteoclast formation by different mechanisms. Bone 11, 53-59.

Schilling, T., Noth, U., Klein-Hitpass, L., Jakob, F. and Schutze, N. 2007. Plasticity in adipogenesis and osteogenesis of human mesenchymal stem cells. Mol Cell Endocrinol 271, 1-17.

Seeman, E. 2003. Bone quality. Osteoporos Int 14 Suppl 5, S3-7.

Singh, S.U., Casper, R.F., Fritz, P.C., Sukhu, B., Ganss, B., Girard, B., Jr., Savouret, J.F. and Tenenbaum, H.C. 2000. Inhibition of dioxin effects on bone formation in vitro by a newly described aryl hydrocarbon receptor antagonist, resveratrol. J Endocrinol 167, 183-195.

Skillington, J., Choy, L. and Derynck, R. 2002. Bone morphogenetic protein and retinoic acid signaling cooperate to induce osteoblast differentiation of preadipocytes. J Cell Biol 159, 135-146.

Slob, W. 2002. Dose-response modeling of continuous endpoints. Toxicol Sci 66, 298-312.

Song, H.M., Nacamuli, R.P., Xia, W., Bari, A.S., Shi, Y.Y., Fang, T.D. and Longaker, M.T. 2005. High-dose retinoic acid modulates rat calvarial osteoblast biology. J Cell Physiol 202, 255-262.

Song, L. and Tuan, R.S. 2004. Transdifferentiation potential of human mesenchymal stem cells derived from bone marrow. FASEB J 18, 980-982.

Sonne, C., Dietz, R., Born, E.W., Riget, F.F., Kirkegaard, M., Hyldstrup, L., Letcher, R.J. and Muir, D.C. 2004. Is bone mineral composition disrupted by organochlorines in east Greenland polar bears (Ursus maritimus)? Environ Health Perspect 112, 1711-1716.

Summer, C.L., Giesy, J.P., Bursian, S.J., Render, J.A., Kubiak, T.J., Jones, P.D., Verbrugge, D.A. and Aulerich, R.J. 1996. Effects induced by feeding organochlorine-contaminated carp from Saginaw Bay, Lake Huron, to laying White Leghorn hens. II. Embryotoxic and teratogenic effects. J Toxicol Environ Health 49, 409-438.

Swanson, H.I. 2002. DNA binding and protein interactions of the AHR/ARNT heterodimer that facilitate gene activation. Chem Biol Interact 141, 63-76.

Thaweboon, S., Thaweboon, B., Choonharuangdej, S., Chunhabundit, P. and Suppakpatana, P. 2005. Induction of type I collagen and osteocalcin in human dental pulp cells by retinoic acid. Southeast Asian J Trop Med Public Health 36, 1066-1069.

Thompson, H.M., Fernandes, A., Rose, M., White, S. and Blackburn, A. 2006. Possible chemical causes of skeletal deformities in grey heron nestlings (Ardea cinerea) in North Nottinghamshire, UK. Chemosphere 65, 400-409.

Tijet, N., Boutros, P.C., Moffat, I.D., Okey, A.B., Tuomisto, J. and Pohjanvirta, R. 2006. Aryl hydrocarbon receptor regulates distinct dioxin-dependent and dioxin-independent gene batteries. Mol Pharmacol 69, 140-153.

Togari, A., Kondo, M., Arai, M. and Matsumoto, S. 1991. Effects of retinoic acid on bone formation and resorption in cultured mouse calvaria. Gen Pharmacol 22, 287-292.

Tuomisto, J.T., Viluksela, M., Pohjanvirta, R. and Tuomisto, J. 1999. The AH receptor and a novel gene determine acute toxic responses to TCDD: segregation of the resistant alleles to different rat lines. Toxicol Appl Pharmacol 155, 71-81.

Turner, C.H. 1998. Three rules for bone adaptation to mechanical stimuli. Bone 23, 399-407.

Wallin, E., Rylander, L. and Hagmar, L. 2004. Exposure to persistent organochlorine compounds through fish consumption and the incidence of osteoporotic fractures. Scand J Work Environ Health 30, 30-35.

Wan, D.C., Siedhoff, M.T., Kwan, M.D., Nacamuli, R.P., Wu, B.M. and Longaker, M.T. 2007. Refining retinoic acid stimulation for osteogenic differentiation of murine adipose-derived adult stromal cells. Tissue Eng 13, 1623-1631.

Van den Berg, M., Birnbaum, L.S., Denison, M., De Vito, M., Farland, W., Feeley, M., Fiedler, H., Hakansson, H., Hanberg, A., Haws, L., Rose, M., Safe, S., Schrenk, D., Tohyama, C., Tritscher, A., Tuomisto, J., Tysklind, M., Walker, N. and Peterson, R.E. 2006. The 2005 World Health Organization reevaluation of human and Mammalian toxic equivalency factors for dioxins and dioxin-like compounds. Toxicol Sci 93, 223-241.

van der Ven, L.T., van de Kuil, T., Leonards, P.E., Slob, W., Lilienthal, H., Litens, S., Herlin, M., Hakansson, H., Canton, R.F., van den Berg, M., Visser, T.J., van Loveren, H., Vos, J.G. and Piersma, A.H. 2009. Endocrine effects of hexabromocyclododecane (HBCD) in a one-generation reproduction study in Wistar rats. Toxicol Lett 185, 51-62.

Wehrli, F.W., Gomberg, B.R., Saha, P.K., Song, H.K., Hwang, S.N. and Snyder, P.J. 2001. Digital topological analysis of in vivo magnetic resonance microimages of trabecular bone reveals structural implications of osteoporosis. J Bone Miner Res 16, 1520-1531.

Wejheden, C., Brunnberg, S., Larsson, S., Lind, P.M., Andersson, G. and Hanberg, A. 2010. Transgenic mice with a constitutively active aryl hydrocarbon receptor display a gender-specific bone phenotype. Toxicol Sci 114, 48-58.

Weston, A.D., Hoffman, L.M. and Underhill, T.M. 2003. Revisiting the role of retinoid signaling in skeletal development. Birth Defects Res C Embryo Today 69, 156-173.

Weston, W.M., Nugent, P. and Greene, R.M. 1995. Inhibition of retinoic-acid-induced gene expression by 2,3,7,8-tetrachlorodibenzo-p-dioxin. Biochem Biophys Res Commun 207, 690-694.

White, S.S. and Birnbaum, L.S. 2009. An overview of the effects of dioxins and dioxin-like compounds on vertebrates, as documented in human and ecological epidemiology. J Environ Sci Health C Environ Carcinog Ecotoxicol Rev 27, 197-211.

Wold, S., Sjöström, M. and Eriksson, L. 2001. PLS-regression: a basic tool of chemometrics. Chemometrics and Intelligent Laboratory Systems 58, 109-130.

Voronov, I., Heersche, J.N., Casper, R.F., Tenenbaum, H.C. and Manolson, M.F. 2005. Inhibition of osteoclast differentiation by polycyclic aryl hydrocarbons is dependent on cell density and RANKL concentration. Biochem Pharmacol 70, 300-307.

Voronov, I., Li, K., Tenenbaum, H.C. and Manolson, M.F. 2008. Benzo[a]pyrene inhibits osteoclastogenesis by affecting RANKL-induced activation of NF-kappaB. Biochem Pharmacol 75, 2034-2044.

Xiong, J. and O'Brien, C.A. 2012. Osteocyte RANKL: new insights into the control of bone remodeling. J Bone Miner Res 27, 499-505.

Yamashita, A., Takada, T., Narita, J., Yamamoto, G. and Torii, R. 2005. Osteoblastic differentiation of monkey embryonic stem cells in vitro. Cloning Stem Cells 7, 232-237.

Yilmaz, B., Seyran, A.D., Sandal, S., Aydin, M., Colakoglu, N., Kocer, M. and Carpenter, D.O. 2006. Modulatory effects of Aroclors 1221 and 1254 on bone turnover and vertebral histology in intact and ovariectomized rats. Toxicol Lett 166, 276-284.

Yonezawa, T., Hasegawa, S., Ahn, J.Y., Cha, B.Y., Teruya, T., Hagiwara, H., Nagai, K. and Woo, J.T. 2007. Tributyltin and triphenyltin inhibit osteoclast differentiation through a retinoic acid receptor-dependent signaling pathway. Biochem Biophys Res Commun 355, 10-15.

Zile, M.H. 1992. Vitamin A homeostasis endangered by environmental pollutants. Proc Soc Exp Biol Med 201, 141-153.

Zile, M.H. 2001. Function of vitamin A in vertebrate embryonic development. J Nutr 131, 705-708.

Zioupos, P. 2005. In vivo fatigue microcracks in human bone: material properties of the surrounding bone matrix. Eur J Morphol 42, 31-41.

Zioupos, P. and Currey, J.D. 1998. Changes in the stiffness, strength, and toughness of human cortical bone with age. Bone 22, 57-66.